Potential of corn distillers dried grains with solubles (DDGS) in diets for turbot (*Scophthalmus maximus*) and gilthead seabream (*Sparus aurata*)

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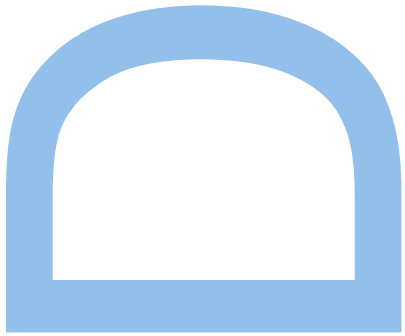

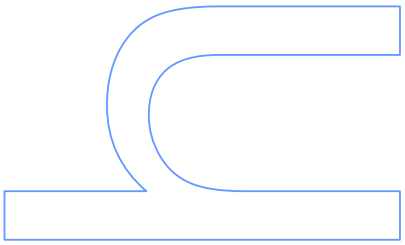

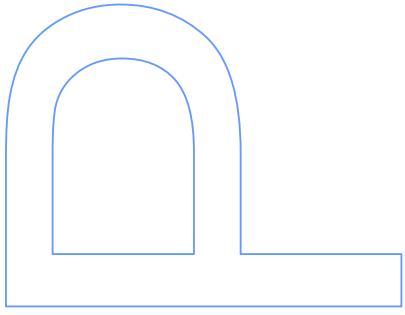

Previous note

This thesis, was made with paragraph 2 of Article 4 of the General Regulation of Third Cycle of Studies, University of Porto and Article 31 of Decree 74/2006, of March 24, with new wording introduced by Decree 230/2009 of September 14, the total utilization of a coherent set of research papers already published or submitted for publication in international journals indexed and peer review, which comprise some of the chapters of this thesis was made. Given that such work was done in collaboration with some other authors, the candidate clarifies that, in all of them actively participated in its design, obtaining, analysis and discussion of results, as well as in preparing its published form.

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List of published papers

Diógenes, A.F., Castro, C., Miranda, A., Oliva-Teles, A., Peres, 2018. Dietary replacement of fishmeal by corn distillers dried grains with solubles (DDGS) in diets for turbot (*Scophthalmus maximus*, Linneaus, 1758) Juveniles. Aquaculture 492, 113-122.

Diógenes, A.F., Castro, C., Carvalho, M., Magalhães, R., Estevão-Rodrigues, T.T., Serra, C.R., Oliva-Teles, A., Peres, 2018. Exogenous enzymes supplementation enhances diet digestibility and digestive function and affects intestinal microbiota of turbot (*Scophthalmus maximus*) juveniles fed distillers' dried grains with solubles (DDGS) based diets. Aquaculture 486, 42-50.

List of manuscript submitted

Diógenes, A.F., Basto, A., Estevão-Rodrigues, T.T., Aires, T., Oliva-Teles, A., Peres, H., 2018. Soybean meal replacement by corn distillers dried grains with solubles (DDGS) and exogenous non-starch polysaccharidases supplementation in diets for gilthead seabream (*Sparus aurata*) juveniles. Aquaculture.

Diógenes, A. F., Teixeira, C., Almeida, E., Skrzynska, A., Costas, B., Oliva-Teles, A., Peres, H., 2018. Effects of dietary tryptophan and chronic stress in gilthead seabream (*Sparus aurata*) juveniles fed corn distillers dried grains with solubles (DDGS) based diets. Aquaculture.

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Abstract

Aquafeed industry faces numerous challenges, including heavy dependence on fish meal (FM) and fish oil (FO); high demand for high protein content feedstuffs; high feedstuffs and energy costs; and market availability of feedstuffs. Search for new and alternative aquafeed ingredients, with adequate nutritional value and nutrient bioavailability, sustainability and safety, has therefore come to the forefront. Plant ingredients and particularly plant by-products from food, fermentation and pharmaceutical industries are promising ingredients. Evaluation of their nutritional value is however required for considering their potential of inclusion in least-cost feed formulas, and to promote the “eco-nutrition” and “eco-efficiency” of aquaculture. However, to accomplish this, it is of utmost importance to gather scientific knowledge on feed quality in order to develop strategies to reduce negative impacts of these novel feed ingredients on growth, feed utilization and welfare of fish. Corn distillers' dried grains with solubles (DDGS) is the main by-product of ethanol production with high potential of production expansion due to the interest on renewable fuels. Improvements in processing and technology of ethanol production, and the precision of diet formulation opened new horizons for ethanol by-products incorporation in aquafeeds. DDGS is readily available and is less expensive than soybean meal (SBM) and other conventional plant ingredients, on a protein-cost basis. However, critical science and knowledge gaps hinder the DDGS incorporation in aquafeeds. DDGS studies have been focused on herbivorous and omnivorous fish species. For carnivorous fish, little research work was done up to now but several nutritional challenges to its use are expected, including high fiber content, low protein content, low protein and energy digestibility and, the presence of phytic acid. Thus, it is of upmost importance to developing strategies to overcome some of the nutritional limitations of its use in carnivorous fish feeds. Under this context, the present work aimed to evaluate the nutritional value of DDGS as an alternative plant ingredient and developed strategies to alleviate some of the limitations of the use of DDGS in diets for two carnivorous marine fish species turbot (*Scophthalmus maximus*) and gilthead seabream (*Sparus aurata*). The effects of dietary inclusion level of DDGS, combined or not with exogenous enzyme complexes or supplemented or not with tryptophan will be evaluated on nutrients digestibility, growth performance, feed utilization efficiency, intestinal function, and antioxidant status of turbot and gilthead seabream juveniles.

In chapter 2 and chapter 4, the dietary inclusion of DDGS in turbot and gilthead seabream were evaluated. In chapter 2, DDGS was used to replace FM at 0, 10, 17.5 and 25% (dry matter basis) in diets for turbot. At the end of the trial, voluntary feed intake was not affected, but growth and feed efficiency linearly decreased with the increase of dietary DDGS level. Whole-body dry matter and protein contents were not affected by diet composition, contrarily of lipid and energy content. The apparent digestibility coefficients (ADCs) of protein and amino acids were similar among diets, while the ADCs of energy decreased with the increase of dietary DDGS level. Digestive amylase and lipase activities in the posterior intestine were lower in fish fed the high DDGS levels diets than the control diet, while proteases activity was not affected by diet. The plasma essay was lower in fish fed DDGS diets, except for plasma glucose, that was not affected. Activity of key enzymes of glycolysis, gluconeogenesis, and lipogenesis was not affected by diet composition, but the activity of alanine aminotransferase increased with the increase of dietary DDGS. Moreover, the oxidative status of liver and intestine was not affected by dietary treatments, but susceptibility to oxidative stress was higher in the intestine than in the liver.

For gilthead seabream (Chapter 4), DDGS was used to replace SBM at 0, 15 and 35% (dry matter basis). Dietary replacement of SBM by DDGS did not affect growth performance, voluntary feed intake, feed efficiency, protein and energy retention and tended to decrease the de cost of fish production (€ per kg of fish). Whole-body composition, hepatosomatic and visceral indexes were not affected by the dietary inclusion of DDGS, though a trend to decrease of lipid, energy and visceral index with the increase of DDGS in the diets was observed. Plasma glucose, protein, albumin, and globulins levels were similar among diets, whereas plasma triglycerides increased, and cholesterol decreased with the increase of dietary DDGS level. Hepatic glycolytic enzymes activities (hexokinase and glucokinase) were similar among treatments, while gluconeogenic (fructose biphosphatase) activity, GDH and ASAT activities decreased with the increase of dietary DDGS level. The hepatic activity of the oxidative stress-related enzymes catalase and superoxide dismutase were not affected by the dietary treatments, but the activity of G6PDH and GR decreased and liver lipid peroxidation (measured as malondialdehyde) increased with the increase of dietary DDGS level.

To overcome one the major limitation of DDGS - its high level of non-starch polysaccharides (NSP), in chapter 3 (turbot) and chapter 4 (gilthead seabream) it was studied the supplementation of DDGS based diets with exogenous enzymes. For turbot (chapter 3) a 25% DDGS based diet was supplemented with two exogenous enzymes

complex (Synergen® or Natugrain®TS; SYN and NAT). Both exogenous enzyme complexes increased the apparent digestibility coefficient (ADC) of dry matter, while the ADC of protein, lipid, and energy were only increased with NAT. Moreover, the ADC of methionine, isoleucine, and aspartic acid were increased with both exoenzymes supplementation, while the ADC of lysine and glycine were increased with SYN and the ADC of arginine and threonine were increased with NAT. Dietary supplementation with SYN or NAT did not affect intestinal pH but increased the activity of lipase and protease in the posterior intestine, while amylase activity was increased only with NAT. Microbiota was also affected by both exoenzymes complexes, increasing its richness and diversity. For gilthead seabream (Chapter 4) a 35% DDGS based diet was supplemented with NAT. Dietary NAT supplementation did not affect growth performance, but increased feed efficiency, nitrogen and energy retention. Dietary NAT supplementation also reduced the fish cost of production that was inclusively lower that that the control SBM based diet. Whole body composition and hepatic key enzymes of intermediary metabolism and oxidative stress were not affected by supplementation with NAT, but it decreased overall hepatic lipid peroxidation.

DDGS unbalance ratio between tryptophan (Trp) and branched-chain amino acids (BCAA) levels may affect synthesis and release of serotonin and may impair the capacity of fish to cope with chronic stress. Thus, in chapter 5, it was studied study the effect of supplementation with Trp (0, 0.13, and 0.25% of the diet) of a 30% DDGS based diets of gilthead seabream maintained at normal and high stock density. Irrespective of the diet, high stocking density reduced growth performance and feed intake, but not feed efficiency. Plasma protein, triglycerides, and cholesterol levels; whole-body lipid, hepatosomatic index, and liver glycogen; hepatic activity of key-enzymes of glycolysis and lipogenesis were also reduced. Moreover, plasma glucose level and hepatic activity of key-enzymes gluconeogenesis were increased. Irrespective of stocking density, diets supplementation with Trp did not affect growth and feed efficiency, but increased hepatic lipase activity and reduced liver lipids, plasma triglycerides and cholesterol levels, and hepatic activity of key-enzymes of amino acid catabolism. Moreover, dietary Trp supplementation restored plasma glucose levels of fish kept at high stocking density to levels similar to that of fish kept at low stocking density.

Overall, for turbot juvenile, FM replacement by DDGS reduced growth performance, impaired overall nitrogen and energy metabolism but did not affect the oxidative status of liver and intestine. However, concomitant supplementation of DDGS based diets with an exogenous carbohydrases complex increased digestibility of dry

matter, protein, energy and some EAA, increased the digestive enzyme activities and affects microbiota, increasing its richness and diversity. Thus, for turbot juvenile, further studies are required to extend the study of the application of exogenous enzymes of DDGS based diets, namely to evaluate its economic feasibility.

For gilthead seabream juveniles, the replacement of SBM by 35% of DDGS did not compromise the growth performance, feed protein and energy utilization efficiency and trend to decrease the fish cost production. The concomitant supplementation with a carbohydrases complex (NAT) did not affect growth performance but improved overall feed utilization efficiency and reduce the fish cost production. Contrarily, Trp supplementation of a 30% DDGS based diets did not affect growth and feed efficiency but seemed to mitigate stress response of gilthead seabream juveniles kept at high stocking density. Further studies are however necessary to further elucidate the role of dietary Trp surplus on the stress response of gilthead seabream.

Overall this thesis demonstrated the high potential of DDGS as an alternative to the traditional plant feedstuffs, as SBM, for gilthead seabream, while for turbot, the potential of DDGS to replace FM is limited. For both species, the potential of use of exogenous enzymes complex, namely carbohydrases, to increase feed utilization efficiency was highlighted.

Keywords: amino acids, by-products, chronic stress, DDGS, digestibility, digestive enzymes, exogenous enzymes, growth performance, intermediary metabolism, microbiota, nutrient utilization, oxidative stress, plant feedstuffs, stocking density.

Resumo

A indústria de alimentos para animais enfrenta inúmeros desafios, incluindo grande dependência de farinha de peixe (FM) e óleo de peixe; alta demanda por alimentos ricos em proteínas; altos custos com rações e energia; e disponibilidade de rações para animais. A busca por alternativas ou novos ingredientes na aquicultura, com valor nutricional adequado e biodisponibilidade de nutrientes, sustentabilidade e segurança, tem, portanto, vindo à tona. Os ingredientes vegetais e particularmente os subprodutos vegetais provenientes de alimentos, fermentação e indústrias farmacêuticas são ingredientes promissores. A avaliação do seu valor nutricional é, no entanto, necessária para considerar o seu potencial de inclusão nas fórmulas alimentares de menor custo e para promover a "eco nutrição" e a "eco eficiência" na aquicultura. No entanto, é de extrema importância reunir conhecimento científico sobre a qualidade do alimento, a fim de desenvolver estratégias para reduzir os impactos negativos desses novos ingredientes no crescimento, na utilização de alimentos e no bem-estar dos peixes. Os grãos secos destilados com solúveis (DDGS) é o principal subproduto da produção do etanol com alto potencial de expansão da produção devido o interesse em combustíveis renováveis. Melhorias no processamento e na tecnologia de produção de etanol e na precisão da formulação de dietas abriram novos horizontes para a incorporação de subprodutos do etanol na aquicultura. O DDGS está prontamente disponível e é mais barato que o farelo de soja (SBM) e outros ingredientes vegetais convencionais, com base no custo de proteína. No entanto, lacunas críticas do conhecimento e da ciência dificultam a incorporação de DDGS na aquicultura. Os estudos de DDGS foram focados em espécies de peixes herbívoros e onívoros. Para os peixes carnívoros, pouco trabalho de pesquisa foi feito até o momento, mas vários desafios nutricionais para seu uso são esperados, incluindo alto conteúdo de fibra, baixo teor de proteína, baixa digestibilidade proteica e energética e presença de ácido fítico. Assim, é de suma importância desenvolver estratégias para superar algumas das limitações nutricionais de seu uso em rações para peixes carnívoros. Sob este contexto, o presente trabalho teve como objetivo avaliar o valor nutricional do DDGS como um ingrediente vegetal alternativo e desenvolver estratégias para aliviar algumas das limitações do uso de DDGS em dietas para dois peixes carnívoros como o rodovalho (*Scophthalmus maximus*) e a dourada (*Sparus aurata*). Os efeitos do nível de inclusão alimentar de DDGS, combinados ou não com complexos enzimáticos exógenos ou suplementados ou não com triptofano, serão avaliados quanto à digestibilidade dos

nutrientes, desempenho de crescimento, eficiência de utilização de alimentos, função intestinal e status antioxidante de juvenis de girinos e dourados.

No Capítulo 2 e no Capítulo 4, a inclusão dietética do DDGS no rodovalho e na dourada foram avaliadas. No capítulo 2, DDGS foi usado para substituir FM a 0, 10, 17,5 e 25% (base de matéria seca) em dietas para rodovalho. No final do ensaio, a ingestão voluntária de ração não foi afetada, mas o crescimento e a eficiência alimentar diminuíram linearmente com o aumento do nível de DDGS na dieta. Os teores de matéria seca e proteína corporal não foram afetados pela composição da dieta, ao contrário do conteúdo lipídico e energético. Os coeficientes de digestibilidade aparente (ADCs) de proteína e aminoácidos foram semelhantes entre as dietas, enquanto os ADCs de energia diminuíram com o aumento do nível de DDGS na dieta. As atividades da amilase digestiva e da lipase no intestino posterior foram menores nos peixes alimentados com dietas ricas em DDGS do que na dieta controle, enquanto a atividade das proteases não foi afetada pela dieta. Os dados plasmáticos foram menores em peixes alimentados com dietas DDGS, com exceção da glicose plasmática, que não foi afetada. A atividade das principais enzimas da glicólise, gliconeogénese e lipogénese não foi afetada pela composição da dieta, mas a atividade da alanina aminotransferase aumentou com o aumento da DDGS na dieta. Além disso, o estado oxidativo do fígado e do intestino não foi afetado pelos tratamentos dietéticos, mas a suscetibilidade ao estresse oxidativo foi maior no intestino do que no fígado.

Para a dourada (Capítulo 4), o DDGS foi utilizado para substituir a SBM a 0, 15 e 35% (matéria seca). A substituição dietética de SBM por DDGS não afetou o desempenho de crescimento, a ingestão voluntária de ração, a eficiência alimentar, a retenção de proteína e energia e tendeu a diminuir o custo de produção de peixe (€ por kg de peixe). A composição corporal total, os índices hepatossomáticos e viscerais não foram afetados pela inclusão dietética da DDGS, embora tenha sido observada uma tendência à diminuição do índice lipídico, energético e visceral com o aumento do DDGS nas dietas. Os níveis plasmáticos de glicose, proteína, albumina e globulinas foram semelhantes entre as dietas, enquanto os triglicerídeos plasmáticos aumentaram e o colesterol diminuiu com o aumento do nível de DDGS na dieta. As atividades das enzimas glicolíticas hepáticas (hexoquinase e glucoquinase) foram semelhantes entre os tratamentos, enquanto a atividade gliconeogênica (frutose bifosfatase), GDH e ASAT diminuíram com o aumento do nível de DDGS na dieta. A atividade hepática das enzimas catalase e superóxido dismutase relacionadas ao estresse oxidativo não foi afetada pelos tratamentos dietéticos, mas a atividade de G6PDH e GR diminuiu e a

peroxidação lipídica do fígado (medida como malondialdeído) aumentou com o aumento do nível de DDGS na dieta.

Para superar uma das principais limitações do DDGS - seu alto nível de polissacarídeos não amiláceos (NSP), no capítulo 3 (rodovalho) e no capítulo 4 (dourada) estudou-se a suplementação de dietas à base de DDGS com enzimas exógenas. Para o pregado (capítulo 3), uma dieta à base de DDGS a 25% foi suplementada com duas enzimas exógenas complexas (Synergen® ou Natugrain®TS; SYN e NAT). Ambos os complexos enzimáticos exógenos aumentaram o coeficiente de digestibilidade aparente (CDA) da matéria seca, enquanto o CDA de proteína, lipídio e energia foram aumentados apenas com a NAT. Além disso, os níveis de ADC de metionina, isoleucina e ácido aspártico aumentaram com a suplementação de exoenzimas, enquanto os CDAs de lisina e glicina aumentaram com o SYN e o ADC de arginina e treonina aumentaram com o NAT. A suplementação dietética com SYN ou NAT não afetou o pH intestinal, mas aumentou a atividade da lipase e protease no intestino posterior, enquanto a atividade da amilase foi aumentada apenas com o NAT. A microbiota também foi afetada por ambos os complexos de exoenzimas, aumentando sua riqueza e diversidade. Para a dourada (Capítulo 4), uma dieta baseada em DDGS de 35% foi suplementada com NAT. A suplementação dietética de NAT não afetou o desempenho do crescimento, mas aumentou a eficiência alimentar, a retenção de nitrogênio e energia. A suplementação dietética de NAT também reduziu o custo de produção do peixe, que foi inclusive menor do que a dieta baseada no controle SBM. A composição corporal total e as enzimas-chave hepáticas do metabolismo intermediário e do estresse oxidativo não foram afetadas pela suplementação com NAT, mas diminuíram a peroxidação lipídica hepática em geral.

A relação de desequilíbrio de DDGS entre os níveis de triptofano (Trp) e aminoácidos de cadeia ramificada (BCAA) pode afetar a síntese e liberação de serotonina e pode prejudicar a capacidade do peixe de lidar com o estresse crônico. Assim, no capítulo 5, estudou-se o efeito da suplementação com Trp (0, 0,13 e 0,25% da dieta) de dietas à base de DDGS a 30% de dourada, mantidas em densidade normal e alta. Independentemente da dieta, a alta densidade populacional reduziu o desempenho de crescimento e o consumo de ração, mas não a eficiência alimentar. Níveis plasmáticos de proteína, triglicerídeos e colesterol; lípido de corpo inteiro, índice hepatossomático e glicogênio hepático; atividade hepática de enzimas-chave da glicólise e lipogénese também foram reduzidos. Além disso, o nível de glicose plasmática e a atividade hepática da enzimas-chave gliconeogénese foram

aumentados. Independentemente da densidade de estocagem, a suplementação de dietas com Trp não afetou o crescimento e a eficiência alimentar, mas aumentou a atividade da lipase hepática e reduziu lipídios hepáticos, triglicérides plasmáticos e níveis de colesterol, e atividade hepática de enzimas-chave do catabolismo de aminoácidos. Além disso, a suplementação de Trp na dieta restaurou os níveis de glicose no plasma de peixes mantidos em alta densidade de estocagem a níveis semelhantes aos de peixes mantidos em baixa densidade populacional.

No geral, para juvenis de rodovalho, a substituição de FM por DDGS reduziu o desempenho de crescimento, metabolismo de nitrogênio e a energia total, mas não afetou o estado oxidativo do fígado e do intestino. No entanto, a suplementação das dietas à base de DDGS com um complexo exógeno de carboidrases aumentou a digestibilidade da matéria seca, proteína, energia e alguns EAA, aumentou as atividades das enzimas digestivas e afetou a microbiota, aumentando sua riqueza e diversidade. Assim, para juvenis de rodovalho, são necessários mais estudos para ampliar o estudo da aplicação de enzimas exógenas de dietas à base de DDGS, a saber, avaliar sua viabilidade econômica.

Para juvenis de dourada, a substituição de SBM por 35% de DDGS não comprometeu o desempenho de crescimento, a eficiência de proteína alimentar e de utilização de energia e a tendência de diminuir a produção de custo de pescado. A suplementação com um complexo de carbohidrases (NAT) não afetou o desempenho do crescimento, mas melhorou a eficiência geral de utilização de ração e reduziu a produção de custo de peixe. Ao contrário, a suplementação com Trp de dietas à base de DDGS 30% não afetou o crescimento e a eficiência alimentar, mas parece mitigar a resposta ao estresse de juvenis de dourada, mantidos em alta densidade populacional. No entanto, são necessários estudos adicionais para esclarecer melhor o papel do excedente de Trp na dieta sobre a resposta ao estresse da dourada.

Palavras-chave: aminoácidos, DDGS, densidade de estocagem, desempenho de crescimento, digestibilidade, enzimas digestivas, enzimas exógenas, estresse crônico, estresse oxidativo, ingredientes vegetais; metabolismo intermediário, microbiota, subprodutos, utilização de nutrientes.

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List of Abbreviations

AA – Amino acid

AAR – Amino acid response

ABW – Average body weight

ADCs – Apparent digestibility coefficients

ALAT – Alanine aminotransferase

ANFs – Anti-nutritional factors

ASAT– Aspartate aminotransferase

BCAA – Branched-chain amino acids

C – Corn

CAT – Catalase

DDGE – Denaturing gradient gel electrophoresis

DDGS – Distiller's dried grains with solubles

DGI – Daily growth index

DP – Digestible protein

DS – distillers' solubles

EAA – Essential amino acid

EDDGS – Corn Ethanol extracted distiller's dried grains with solubles

ER – Energy retention

€ – Feeding cost

FBPase - Fructose-1,6-bisphosphatase

FBW – Final body weight

FE – Fed efficiency

FI – Feed intake

FM – Fishmeal

G6PDH – Glucose-6-phosphate dehydrogenase

GDDY – Grain distillers' dried yeast

GDH – Glutamate dehydrogenase

GK – Glucokinase

GPX – Glutathione peroxidase

GR – Glutathione reductase

HIS – Hepatosomatic index

HK – Hexokinase

HPDDGS – High protein distiller's dried grains with solubles

HSD – High stocking densities

IBW – Initial body weight

IGF-I – Insulin-like growth factor 1

L – Lipids

LEDDGS – Lid extracted distiller's dried grains with solubles

LPO – Lipid peroxidation

LSD – Low stocking densities

MDA – Malondialdehyde

MFM – Menhaden fish meal

mU – milliunit

NAT – Natugrain®TS

nmol – nanomol

NPS – Non-starch polysaccharide

NR – Nitrogen retention

OTUs – Operational taxonomic units.

P – Protein

PER – Protein efficiency ratio

PK – Pyruvate kinase

PP – Plant protein

RD – Reference diet

ROS – Reactive oxygen species

SBM – Soybean meal

SDDGS – Sorghum distiller's dried grains with solubles

SIMPER – Similarity percentage within group replicates

SOD – Superoxide dismutase

SYN – Synergen®

TAG – Triglycerides

TOR – Target of rapamycin

TRP – Tryptophan

U – Unit

VI – visceral index

WDDGS – Whiskey distiller's dried grains with solubles

WG – Weight gain

WG – Wet grains

WtDDGS – Wheat distiller's dried grains with solubles

Chapter 1

General Introduction

1.1 Aquaculture production

Aquaculture is defined by the United Nations Food and Agriculture Organization (FAO) as "farming of aquatic organisms including fish, mollusks, crustaceans and aquatic plants in both coastal and inland areas involving interventions in the rearing process to enhance production". The stagnation of wild fisheries and overexploitation of popular marine species, combined with a growing human demand for high-quality protein has encouraged the domestication of fish species for aquaculture production (FAO, 2016). In fact, aquaculture is considered to be the fastest growing food-producing activity in the world (FAO, 2016), outpacing human population growth, therefore making an important contribution to poverty alleviation and social well-being. Aquaculture share of total fishery production for human consumption is expected to grow from 44% in 2013-2015 to 52% in 2025 (Figure 1).

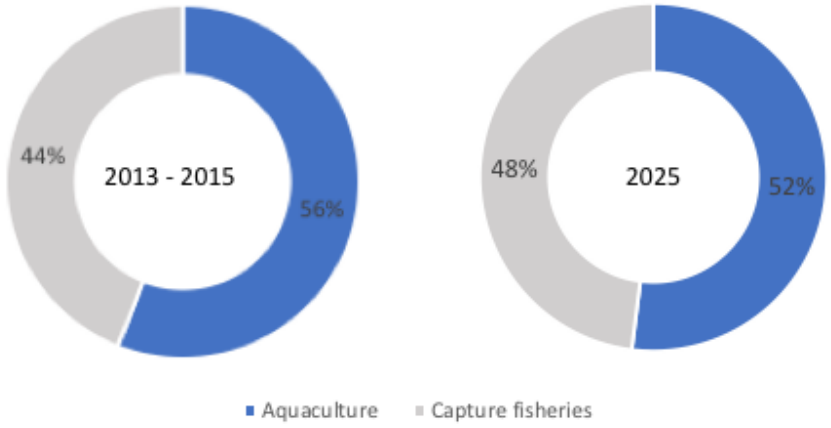

Figure 3. Relative contribution of aquaculture and captures to world fish supply for human consumption. (FAO, 2016).

By 2014, the number of aquaculture species produced around the world was circa 580 of which 362 were finfish (FAO, 2016). Production of these species is done according to different rearing conditions, densities, and water salinities and the world distribution of aquaculture production and species produced vary among regions and is dependent of economical, technological, and environmental factors.

The importance of aquaculture as a source of healthy foods for humans is undeniable. Fish is recognized as unique towards achieving healthy diets, due to its high-quality protein, micronutrients and lipids, which are naturally rich in long-chain omega-3 polyunsaturated fatty acids. Because of that, and along with the increase of world population, fish consumption per capita has been increased. This is particularly true for European countries that averaged 24.9 kg of fish or seafood consumption per capita per

year (6 kg more than in the rest of the world; www. ec.europa.eu/commission). Almost all fish produced in aquaculture are destined for human consumption.

According to FAO (2016) aquaculture production amounted to 73.8 million tons in 2014. However, in the same year, 21 million tonnes was destined for non-food products, of which 76 percent was reduced to fishmeal and fish oil (FAO, 2016). The projections point towards a further increase in the demand for seafood products to 2030, which will require a conjoined effort to increase aquaculture production and to decrease the use of fisheries products for non-food purposes.

1.2 Aquafeeds

Aquaculture production is characterized by being mostly intensive in developing countries, but the feed is widely regarded as becoming a major constraint to the growth of aquaculture production. Indeed, global production from feed-dependent aquaculture increased over fourfold, from 12.2 to 50.7 million tonnes (FAO, 2016). Considering that aquafeed industry is traditionally based on fishery-derived products – fish meal (FM) and fish oil (FO), environmental, social and economic issues compromise the sustainable development of aquaculture. concerns arise. The commitment to increase aquaculture production will require the reduction of the use of fisheries products, and the optimization of feed production and on-farm feed management practices in aquaculture.

The optimal dietary protein level required for the optimal fish growth in aquaculture production is 30-55% on a dry weight basis, a value that is higher than that of farm animals (NRC, 2011). FM has been used as the major protein source in aquafeeds due to its high protein content, excellent amino acid (AA) profile, high nutrient digestibility, general lack of anti-nutrients, relatively low price (until now), and wide availability (Gatlin et al., 2007). The incorporation of FM in aquafeeds usually leads to good growth and feed efficiency due to the aforementioned nutritional characteristics and its high palatability, which stimulates feed uptake (Miles and Chapman, 2005). According to Taco and Metian (2015), aquaculture sector consumed 68% of the total global FM production in 2012, mainly for feeding carnivorous fish species such as salmon, trout, sea bass, seabream, yellowtail and others. However, it is expected that access to FM will become increasingly limited due to decreased availability of this finite wild-harvest resource and the significant increase of global aquaculture production (Gatlin et al. 2007; Tacon and Metian, 2015). Therefore, it is of economic and environmental utmost importance to replace FM with more sustainable and renewable protein sources in aquafeeds.

Under the actual panorama, search for more affordable and more available high-quality alternatives to FM has become a priority, especially for carnivorous fish species. Alternative protein sources consist mainly of plant feedstuffs (e.g., by-products of soybean meal, cottonseed meal, rapeseed meal, corn and wheat gluten; Trushenski et al., 2006; Gatlin et al., 2007), that are considered more sustainable, highly available, and lower-cost protein sources. However, comparatively to FM alternative ingredients have several disadvantages, such as low protein content, AA unbalances, presence of anti-nutritional factors (ANFs), low palatability and high proportion of fiber and non-starch polysaccharides, which must be taken into consideration when considering an effective FM replacement without impairing fish growth, feed utilization, welfare and health (Lim et al., 2008).

Plant-protein sources are already used as complementary to FM in carnivorous fish diets. However, high dietary incorporation levels of plant ingredients have only been achieved with limited success. Indeed, plant ingredients are often associated with lower feed intake, feed efficiency, digestibility and growth, and to induce intestinal disorders, particularly in carnivorous fish species, such as turbot, *Scophthalmus maximus* (Regost et al., 1999), rainbow trout, *Oncorhynchus mykiss* (Refstie et al., 2000), cobia, *Rachycentron canadum* (Chou et al., 2004), yellowtail, *Seriola dumerili* (Tomas et al., 2005), cuneate drum, *Nibea miichthioides* (Wang et al., 2006) or sharpsnout seabream, *Diplodus puntazzo* (Hernandez et al., 2007) and salmonids (Venold et al., 2012; Hartviksen et al., 2014).

Among plant protein sources, the most cost-effective and more widely used as an alternative to FM is soybean meal (SBM) (Gatlin et al., 2007; Bakke-McKellep and Refstie, 2008; Ayadi et al., 2011). Indeed, SBM has a favorable protein content, relatively well-balanced amino acid profile, acceptable digestibility, consistent composition and quality, and it is readily available in the market. A number of studies have shown that proper replacement of fishmeal with SBM does not affect growth performance and feed utilization of fish and crustaceans (Robaina et al. 1995, 1998; Kissil et al. 2000; Pereira and Oliva-Teles, 2002; Martinez-Llorens et al., 2007; Ding et al. 2015a, b). However, SBM contains ANFs, such as protease inhibitors, lectins, phytohaemagglutinin, phytic acids, saponins, phytoestrogens, and antivitamins, which are major drawbacks (Hajra et al., 2013). Associated with the rise in price of SBM (118% since 1998; Cummins et al., 2013) and the “food-feed competition”, it is necessary to find innovative aquafeed ingredients, particularly those that are not used directly for human consumption, such as the by-products obtained from food, fermentation, and pharmaceutical industries. To

accomplish this, it is of utmost importance to gather scientific knowledge on the nutritional quality of these potential alternative ingredients and to develop strategies to reduce potential negative impacts of these novel feed ingredients on fish growth, feed utilization, welfare, and health.

1.3 Distiller's Dried Grains with Solubles

In recent years, the rapid increase of ethanol production (Figure 2) mainly from coarse grains (i.e., wheat and corn), and the subsequent production of by-products, mostly distiller's dried grains with solubles (DDGS), has led to increased availability, improved quality, and higher use of this commodity in livestock diets. Also, in Europe, production of ethanol has sharply increased, along with its co-products, the non-genetically modified DDGS to the domestic feed market (Figure 3).

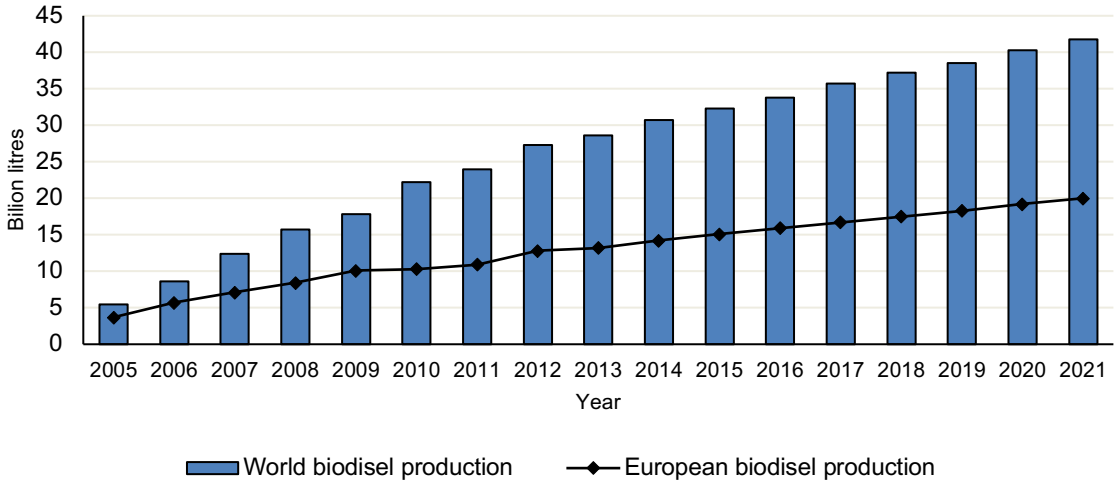

Figure 2. Projected development of the world and European biodiesel production. (Adapted from: OECD and FAO secretariats, 2012)

According to Alagón et al. (2016), DDGS is the most important by-product of the bioethanol manufacture industry, representing 0.3 tonnes per ton of processed cereal. Corn dry grind process (Figure 4) requires several key steps and has become the predominant method for fuel ethanol production, due to the lower investment and operational requirements, and the advances in fermentation technology (Belyea et al., 2004; Rosentrater 2011). Corn dry grind process includes: grain receiving, distribution, storage, cleaning, grinding, cooking, saccharification, fermentation, distillation, ethanol storage and loadout, centrifugation, and coproduct drying.

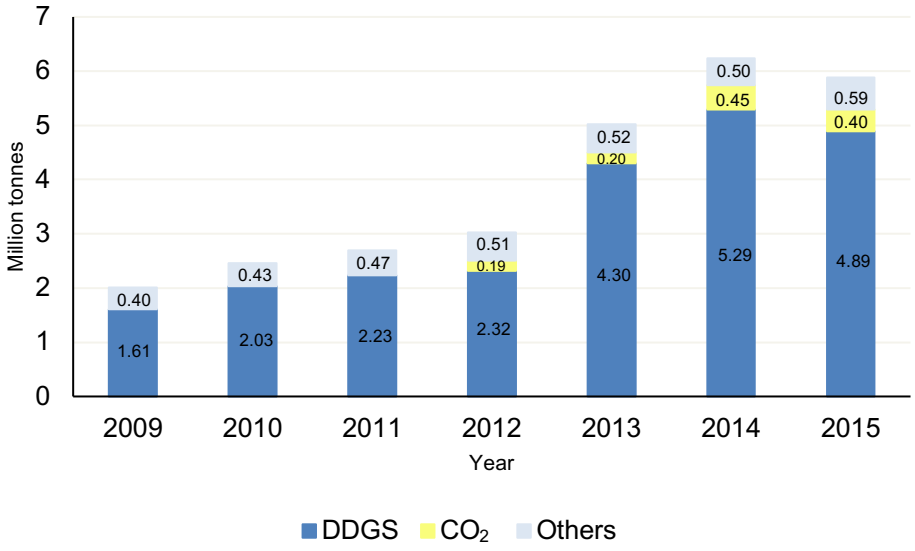

Figure 3. Annual production of co-products of EU renewable ethanol production (Source: ePURE-European Renewable Ethanol)

Briefly, the DDGS production starts with the gridding of whole grain of corn by hammer mills into a course powder with a mean particle diameter of approximately 1 mm (Rausch et al., 2005). After grinding, the corn flour is mixed and/or cooked with water, where the corn starch is converted into sugars by enzymes and fermented into ethanol and carbon dioxide by yeast (Liu, 2012). During this process, starch is prepared for fermentation: the pH is adjusted, the enzyme that breaks-off the long starch polymers into short chains (α-amylase) is added and then the mixture is heated up allowing starch gelatinization and so increasing the asses of α-amylase to starch (Rosentrater, et al., 2012). Then the mixture is cooled and transferred to fermentation tanks, and yeast (*Saccharomyces cerevisiae*) is added to convert glucose into ethanol and carbon dioxide (theoretically 1 g of glucose yields 0.51 g ethanol and 0.49 g carbon dioxide). The pure ethanol vapor is removed and collected. This step results in some non-volatile components called “whole stillage” (Bothast and Schlicher, 2005), that contains fiber, oil, protein, other unfermented components of the corn, and yeast cells. The centrifuging of whole stillage, result in a solid fraction, called wet distillers’ grain, and a liquid fraction (Kim et al., 2008). A significant portion of the liquid fraction is concentrated through multiple effect evaporators to produce a syrup (Ganesan et al., 2006). The combination of the syrup and the wet distillers’ grains with solubles is then dried to produce dried distillers’ grains with solubles (DDGS) (McAloon et al., 2000).

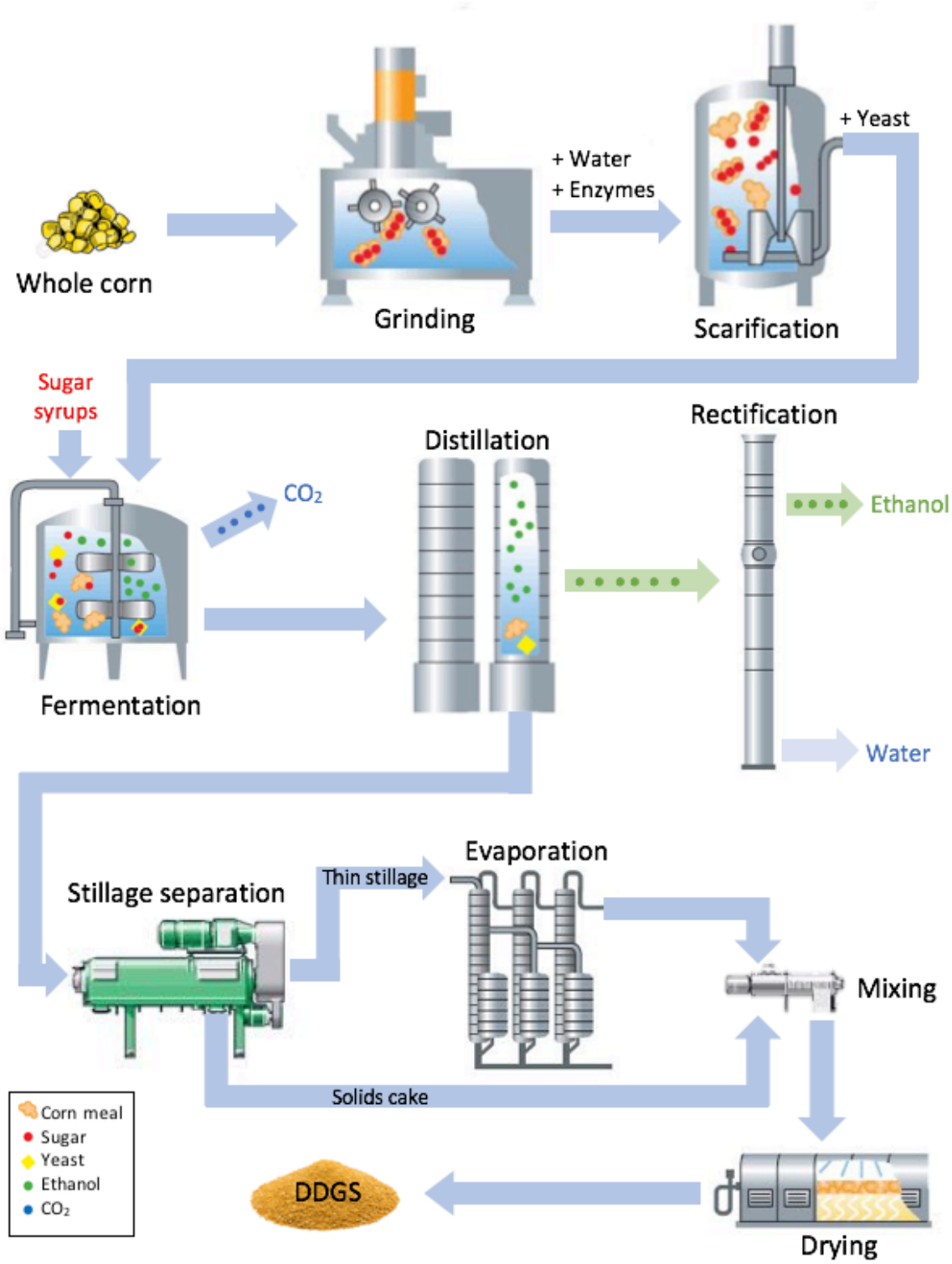

Figure 4. Schematic diagram of the production process of bioethanol and DDGS from corn. (Adapted from Crop Energies, AG Mannheim, 2011).

As aforementioned, DDGS is the non-fermentable component of ethanol process, and its nutrient composition is almost 3 times more concentrated than the original grain (except for the starch fraction) thus containing higher protein, lipid, ash, and fiber levels (Rosentrater and Muthukumarappan, 2006; Liu, 2011). Protein content of corn DDGS (denominated as DDGS from now onward) ranges from 26.3 to 34.0%, fat from 8.8 to 18.5%, neutral detergent fiber from 26.5 to 54.1% (Spiehs et al., 2002; Batal and Dale, 2006; Øverland et al., 2013), and starch up to 5.1% (Belyea et al., 2004), with little presence of anti-nutritional factors (ANFs) commonly found in most plant protein sources (Table 1). DDGS presents some advantages such as price, which is lower than that of FM and plant feedstuffs and the presence of a fair amount of yeast that may act as feed stimulant or probiotic and may have some immunological proprieties. However, DDGS has several disadvantageous characteristics, such as moderate protein content, AA unbalances, high proportion of fiber and non-starch polysaccharide (NSP) levels, and low availability of phosphorus, which must be taken into consideration for an effective DDGS use without impairing growth, feed utilization, welfare, and health of fish (Belyea et al., 2004).

Although DDGS nutrient composition increased about threefold over that of the original grain, the amino acid (AA) composition (relative to total AA) is not substantially improved over the grain. According to Liu (2012), DDGS AA profile is similar to that of corn even though fermentation yeast may somehow affect it. As seen in Table 2, lysine content varies from 0.29% to 1.24% (Øverland et al., 2013; Magalhães et al., 2015); methionine from 0.18% to 0.72% (Øverland et al., 2013; Magalhães et al., 2015); threonine from 0.37% to 1.28% (Øverland et al., 2013; Magalhães et al., 2015), tryptophan from 0.19% to 0.27% (Spiehs et al., 2002; Belyea et al., 2004). In particular, the lysine and methionine levels are low in DDGS relatively to the amino acid requirements of animals. Thus, the balance of dietary essential amino acids (EAA) of DDGS-based diets need to be restored with combination with other ingredients and/or with the addition of synthetic EAA (Welker et al., 2014b).

Table 1. Gross composition of different sources of corn DDGS (% dry matter).

	Spiehs et al. (2002)[1]	Belyea et al. (2004)	Batal and Dale (2006)	Kim et al. (2008)	Han and Liu (2010)	Øverland et al. (2013)	Magalhães et al. (2015)[2]	
Proximate composition							a	b
Dry matter	85.6 – 91.9 (88.9)	-	86.0	88.9	-	95.6	89.2	88.7
Crude protein	26.3 – 34.0 (30.2)	31.3	27.0	27.3	-	27.5	30.4	29.4
Crud lipids	9.2 – 12.6 (10.9)	11.9	8.8	14.5	-	18.5	11.8	12.8
Ash	5.2 – 6.7 (5.8)	4.6	4.4	4.7	-	3.6	4.7	4.9
Starch	-	5.1	-	-	-	5.3	0.5	2.9
Crude fiber	7.1 – 9.7 (8.8)	10.2	6.6	-	-	-	7.2	7.8
Acid detergent fiber	7.0 – 25.4 (16.1)	17.2	-	-	-	-	14.6	13.8
Neutral detergent fiber	30.0 – 54.1 (41.5)	-	-	-	-	26.5	42.4	39.3
Essential amino acids								
Arginine	0.92 – 2.17 (1.20)	1.05	1.09	1.40	1.29	0.50	1.74	1.69
Histidine	0.61 – 0.82 (0.76)	0.70	0.69	0.80	0.91	0.30	0.89	0.83
Isoleucine	1.00 – 1.17 (1.12)	1.52	0.97	1.10	1.03	0.37	1.13	1.11
Leucine	2.97 – 3.81 (3.55)	2.43	3.05	3.30	3.50	1.05	3.66	3.51
Lysine	0.53 – 0.94 (0.85)	0.77	0.71	1.00	1.04	0.29	1.01	1.24
Methionine	0.49 – 0.69 (0.55)	0.54	0.54	0.60	0.72	0.18	0.72	0.67
Phenylalanine	1.27 – 1.57 (1.47)	1.64	1.31	1.40	1.50	0.46	1.33	1.48
Threonine	0.98 – 1.21 (1.13)	1.01	0.96	1.10	1.17	0.37	1.28	1.27
Tryptophan	0.19 – 0.27 (0.25)	0.19	0.2	0.20	-	-	-	-
Valine	1.39 – 1.56 (1.50)	1.63	1.33	1.50	1.56	0.51	1.65	1.57
Non-essential amino acids								
Alanine	-	-	1.78	1.90	2.07	0.65	2.33	2.20
Aspartic acid	-	-	1.75	1.70	1.97	0.64	2.11	2.01
Cysteine	-	-	0.56	0.50	0.57	0.19	-	-
Glutamic acid	-	-	3.49	3.30	5.48	0.34	4.89	4.66
Glycine	-	-	-	1.10	1.19	1.66	1.24	1.17
Proline	-	-	1.99	2.00	2.19	0.70	2.55	2.46
Serine	-	-	1.09	1.20	1.45	0.47	1.70	1.61
Tyrosine	-	0.76	0.96	1.20	1.02	0.32	1.57	1.60

[1]Spiehs et al. (2002); range (mean).
[2]Magalhães et al. (2015) used two different DDGS sources: [a] Biocarburantes de Castilla y Leon, Spain and [b] Pannonia Gold®, Hungry. The DDGS supplied by Pannonia Gold® is the one used in this thesis.

DDGS is readily available in the market, and on a protein-cost basis it is less expensive than conventional plant ingredients, thus being a candidate for fishmeal and plant feedstuff replacement in aquaculture feeds. However, the main problem of using this ingredient in feed formulation is the high variability of nutrient concentration among different DDGS sources (Liu, 2012; Robinson and Li, 2008). DDGS composition varies according to the cereals used for its production, and within a single cereal source it varies over time, due to a number of factors, including the two processes of DDGS production, wet grains (WG) and distillers' solubles (DS) proportions (Singh et al., 2001). Mixing WG and DS prior to drying can be not well controlled; therefore, variation in the proportion of WG to DS can also contribute to nutrients variation in DDGS (Belyea et al., 2004). In fact, differences in the composition of DDGS can be observed in different studies (Table 2).

According to US Grain Council (2012), DDGS has been mainly used in farm animal feeds. Even though the majority of DDGS produced in the USA has been used in ruminant feeds, as DDGS is high in digestible energy, protein, and phosphorus contents, it has also become an economical and widely used ingredient in swine and poultry diets (Stein and Shurson, 2009). However, critical scientific knowledge gaps have hindered the incorporation of DDGS in aquafeeds. Due to some nutritional limitations (high fiber content, moderate protein content, low protein and energy digestibility, and phytic acid content) the incorporation of DDGS in aquafeeds has to be critically evaluated in order to ensure good growth performance, feed utilization efficiency, and well-being of fish. However, except for its fiber and phytic acid contents, DDGS does not contain other ANFs as those found in plant protein sources such as soybean meal, rapeseed meal, or cottonseed meal, and that may interfere with fish performance and health (Gatlin et al., 2007; Krogdahl et al. 2010; Oliva-Teles et al. 2015). According to Shurson (2012) less than 1% of total DDGS produced is currently being used in aquaculture feeds.

To our knowledge, studies evaluating the use of DDGS in fish are limited to a few fish species, such as channel catfish, *Ictalurus punctatus* (Tidwell et al., 1990; Webster et al., 1991, 1992, 1993; Robinson and Li, 2008; Lim et al., 2009; Li et al., 2010, 2011a); European seabass, *Dicentrarchus labrax* (Magalhães et al., 2015); hybrid striped bass, *Morone chrysops* × *M. saxatilis* (Trushenski and Gause, 2013); hybrid tilapia, *Oreochromis niloticus* × *Oreochromis aureus* (Welker et al., 2014a); hybrid tilapia, *O. niloticus* × *O. aureus* (Chatvijitkul et al., 2016); milkfish, *Chanos* (Mamauag et al., 2017); meagre, *Argyrosomus regius* (Magalhães et al., 2015); Nile tilapia, *Oreochromis niloticus*

(Wu et al., 1997; Abo-State et al., 2009; Webster et al., 2016); olive flounder, *Paralichthys olivaceus* (Rahman et al., 2015); rainbow trout, *Oncorhynchus mykiss* (Cheng and Hardy., 2004; Øverland, 2013; Hauptman et al., 2014); sunshine bass, *Morone chrysops* × *Morone saxatilis* (Thompson et al., 2008); yellow perch, *Perca flavescens* (Schaeffer et al., 2011); rainbow trout, *Oncorhynchus mykiss* (Stone et al., 2005) (Table 3).

For freshwater omnivorous fish, DDGS has been considered a promising alternative protein source, and it may be incorporated up to 30% in diets for channel catfish (Prachom et al., 2013) and tilapia (Lim and Yildirim-Aksoy, 2008; Li et al., 2011b). So far, studies performed on the potential use of DDGS in carnivorous species are limited to a few studies with rainbow trout (Øverland et al., 2013; Welker et al., 2014b), olive flounder (Rahman et al., 2015), European seabass and meagre (Magalhães et al., 2016). Øverland et al. (2013) reported that total replacement of a typical plant-based diet by DDGS for rainbow trout led to higher voluntary feed intake and weight gain and feed efficiency. The incorporation of DDGS in olive flounder diet is limited to 13% to assure appropriate growth and feed efficiency (Rahman et al., 2015). Likewise, in rainbow trout it was concluded that DDGS could be included in the diets only up to 10%, even if supplemented with essential amino acids or phytase (Stone et al., 2005; Barnes et al., 2012). According to Welker et al. (2014b) high levels of indigestible structural fiber limit the incorporation of DDGS in diets of rainbow trout to approximately 10-20%. Indeed, event thought some treatments have been used to improve protein and energy digestibility of plant-based aquafeeds, such as hydrothermal treatments and fractionation of crops (Glencross et al., 2012; Castillo and Gatlin, 2015).

Table 2. Studies performed with several aquaculture species using DDGS for replacement dietary ingredients.

Species	HabitatTrophic level[1]	IBW (g)[2]	DDGS Type[3]	DDGS Prot and L content (%DM)[4]	Ingredient replaced (%DM)[5]	Diet Prot and L content (%DM)	DDGS substitution levels (%DM)	Reference
Channel Catfish, *Ictalurus punctatus*	FW 4.2±0.3	1.5	DDGS	-	SBM/C	32%P	20%	Tidwell et al. (1990)
		10.0	DDGS	-	PP	36%P	35%	Webster et al. (1991)
		11.0	DDGS	-	FM	36%P	35%	Webster et al. (1992)
		48.0	DDGS	-	SBM	-	up to 20%	Robinson and Li (2008)
		13.3	DDGS	-	SBM/C	-	up to 20%	Lim et al. (2009)
		86.9	DDGS	-	SBM/C	32%P, 6%L	30%	Zhou et al. (2010)
		9.1	DDGS	-	FM	28%P, 5%L	30%	Li et al. (2011a)
		9.1	EDDGS	-	FM	28%P, 5%L	up to 30%	Li et al. (2011a)
Hybrid tilapia, *O. niloticus* × *O. aureus*	FW 2.1±0.0	6.0	LEDDGS	31%P, 6%L	SBM	36%P	30%	Chatvijitkul et al. (2016)
Hybrid tilapia, *O. niloticus* (♀) × *O. aureus* (♂)	FW 2.0±0.0	3.7	DDGS	31.1%P, 10.5%L	SBM/C	30%P	30%	Welker et al. (2014a)
			DDGS	28.9%P, 4.3%L				
			DDGS	28.9%P, 11.6%L				
			WDDGS	30.7%P, 12.6%L				
			SDDGS	36.6%P, 7.6%L				
			WtDDGS	37.8%P, 5.8%L				
Nile tilapia, *Oreochromis niloticus*	FW 2.0±0.0	0.5	DDGS	-		32%P, 6%L	50-55%	Wu et al. (1997)
		2.0	DDGS	-	SBM	35%P	50%	Abo-State et al. (2009)
		2.6	DDGS	26.5%P, 9%L	MFM/SBM	35%P	up to 45%	Webster et al. (2016)
Rainbow trout, *Oncorhynchus mykiss*	FW 4.1±0.3	300.0	DDGS GDDY	-	RD			Hauptman et al. (2014)
		22.1	DDGS GDDY	-	FM	42%P, 20%L	25%-37.5%	Hauptman et al. (2014)
		49.8	DDGS	-		44%P	15%	Cheng and Hardy (2004)
		143.0	DDGS	27,5%P, 18.5%L	PP	35%	100%	Øverland et al. (2013)
		144.0	HPDDGS	44.7% P, 5.4%L	PP	43%	100%	Øverland et al. (2013)

Sunshine bass, *Morone chrysops* × *M. saxatilis*	FW 4.0±0.7	15.9	DDGS GDDY	42%P, 6.5% L	FM	40%P, 15%L	7.5% -15%.	Gause and Trushenski, (2011a)
		4.4	DDGS GDDY	42%P, 6.5%L	FM	40%P, 15%L	75%	Gause and Trushenski, (2011b)
Yellow perch, *Perca flavescens*	FW 3.7±0.2	19.1	DDGS	27,9%P, 11.5%L	SBM	30.1%P, 16.7%L	40%	Schaeffer et al. (2011)
Rainbow trout, *Oncorhynchus mykiss*	Marine 4.1±0.3	21.0	DDGS	31%P, 13%L	FM	45%P, 16%L	18%	Stone et al. (2005)
Olive flounder, *Paralichthys olivaceus*	Marine 3.7±0.6	11.6	DDGS	21.5%P, 4.5%L	PP	50%P	13%	Rahman et al. (2015)
European seabass, *Dicentrarchus labrax*	Marine 3.5±0.5	206.0	DDGS DDGS	30.4%P, 11.8%L 29.4%P, 12.8%L	RD	45%P, 16%L	30% 30%	Magalhães et al. (2015)
Meagre, *Argyrosomus regius*	Marine 4.3±0.8	78.8	DDGS DDGS	30.4%P, 11.8%L 29.4%P, 12.8%L	RD	45%P, 16%L	30% 30%	Magalhães et al. (2015)
Milkfish, *Chanos chanos*	Marine 2.4±0.2	3.7	DDGS	31%P, 8%L	SBM	35%P, 6%L	45%	Mamauag et al. (2017)
Hybrid Striped Bass *Morone chrysops* × *M. saxatilis*	Marine 4.7 ±0.2	43.4	DDGS	30.4%P, 7.8%L	FM	40%P, 15%L	33%	Trushenski and Gause (2013)

[1]Source: http://www.fishbase.org. FW: freshwater
[2]IBW (g): Initial body weight
[3]DDGS: Corn DDGS; EDDGS: Corn Ethanol extracted DDGS; LEDDGS: Lid extracted DDGS; WDDGS: whiskey DDGS; SDDGS: sorghum DDGS; WtDDGS: wheat DDGS; GDDY: grain distillers' dried yeast; DDGS; HPDDGS: high protein DDGS
[4] P: protein; L: lipids
[5] SBM: soybean meal; C: corn; PP: mixture of the plant protein; FM: fishmeal; RD: reference diet; MFM: menhaden fish meal
[6] DP: digestible protein

The main constraint to higher dietary DDGS inclusion levels is its high content of soluble fiber, which cannot be digested by fish, due to the lack of specific digestive non-starch carbohydrases or, if they have, its concentration is very low (Krogdahl et al., 2005). Indeed, event thought some treatments have been used to improve protein and energy digestibility of plant-based aquafeeds, such as hydrothermal treatments and fractionation of crops. Supplementation of diets with exogenous enzymes may be a strategy for improving the nutritional value of high NSP in plant ingredients, including DDGS (U.S. Council, 2012). The main function of exogenous supplementation of carbohydrases in plant based-diets is to break-down the NSP complex that monogastric animals are not capable of hydrolyzing using their own digestive enzymes (Castillo and Gatlin, 2015).

The use of exogenous enzymes as feed additives has been extensively studied in poultry and pig feed industry, being now a common practice to reduce the anti-nutritional effects of NSP and phytic acid (Adeola and Cowieson, 2011; U.S. Grains Council, 2012; Castillo and Gatlin, 2015). Indeed, for monogastric terrestrial animals it was observed that exoenzymes may improve DDGS nutritional value (Emiola et al., 2009). For poultry and swine production it has been observed that digestibility and growth performance may be enhanced by supplementation of DDGS-based diets with NSP-hydrolyzing enzymes (Spenser et al., 2007; Emiola et al., 2009) as well as with proteases (Bandegan et al., 2009; Adebiyi and Olukosi, 2015). However, for aquaculture species, no scientific evidence exists regarding the potential use of exogenous enzymes on the nutritive value of DDGS.

Use of exogenous enzymes for aquaculture species has been centered on phytase (Oliva-Teles et al., 1998; Debnath et al., 2005; Zhu et al., 2014; Verlhac-Trichet et al., 2014). In recent studies, however, it was observed that diets for gilthead sea bream, *Sparus aurata,* and striped bass supplementation with exogenous enzymes enhanced dietary nutrients utilization and decreased water pollution (Kolkovski et al., 1993; Papatryphon et al., 1999). Studies regarding the use of carbohydrases are still limited and with inconclusive results, depending on the type and inclusion level of plant feedstuffs level, exoenzymes used, and water temperature at which fish are held (Castillo and Gatlin, 2015). One of the first studies demonstrating that supplementation of plant-based diets with exoenzymes improved feed utilization efficiency and growth in fish was done with Atlantic salmon (Carter et al., 1994). Some recent studies also demonstrated beneficial effects of carbohydrases supplementation of plant-based diets in different fish species (Yildirim and Turan, 2010; Ghomi et al., 2012; Goda et al., 2012; Zhou et al.,

2013; Zamini et al., 2014) while other studies did not observe such effects (Ogunkoya et al., 2006; Yigit and Olmez, 2011; Dalsgaard et al., 2012). However, in a recent review, Castillo and Gatlin (2015) concluded, based on the limited number of studies conducted with fish, that exogenous carbohydrases supplementation to plant-based diets seems to improve nutrient digestibility and reduce nutrient excretion.

One of the possible limitations of DDGS use in aquafeeds that may deserve special attention is its low tryptophan (Trp) content relatively to branched-chain amino acids (BCAA). As Trp and BCAA share the same intestinal transporter, low levels of Trp associated with high levels of leucine, may cause amino acids imbalance between these two amino acids (Fernstrom, 2012). For fish, Trp is required not only for protein deposition but also for various metabolic functions such as the synthesis of insulin-like growth factor 1 (IGF-I), regulation of appetite, glucose homeostasis and immune function (Pérez-Sánchez and Le Bail 1999; Le Floc'H and Seve 2007; Matte et al., 2011, Yao et al., 2011). Thus, DDGS based diets may compromise plasma and brain Trp levels, and so may condition the synthesis of important metabolites such as serotonin and melatonin (Lepage et al., 2002). Trp is also the precursor for several compounds, including 5-HT (serotonin), a neurotransmitter that is synthesized via Trp hydroxylase. Serotonin, in turn, can be converted to melatonin, via N-acetyltransferase and 5-hydroxyindole-O-methyltransferase (NRC, 2011). Indeed, Trp is the only precursor of serotonin and melatonin (Lepage et al., 2005; Martins et al., 2013), which are known to be implicated in the control of agonistic behavior, stress responses, endocrine functions, antioxidant and immune responses in animals (Winberg and Thörnqvist, 2016; Hoseini et al., 2017). Thus, limited Trp availability may be a concern, particularly under chronic stress conditions. Dietary supplementation of Trp in DDGS based diets may be an effective strategy for increasing serotonin and melatonin levels in the organism, with the subsequent physiological benefits.

1.4 Turbot

Turbot (*Scophthalmus maximus*) is a demersal fish native to marine or brackish waters of the Northeast Atlantic Sea: throughout the Mediterranean and along the European coasts to Arctic Circle (Figure 5). Turbot is a species of flatfish of the family *Scophthalmidae* and has an almost circular body. It has both eyes on the right side, without scales in the tegument but with large bony tubercles (Muus and Nielsen, 1999). Wild turbot adults live on sandy, rocky or mixed bottoms, rather common in brackish waters. Turbot feeds mainly on other bottom-living fishes (sand-eels, gobies, etc.) and also, to a lesser extent, on larger crustaceans and bivalves (Murua and Saborido-Rey, 2003). Turbot is a very high market value species, and it is one of the major marine fish produced in Europe and Asia due to its appreciated flesh, fast growth rate, well-established production techniques, and tolerance to intensive production conditions. Intensive production of this species is now a well-controlled process, including all the different phases of production (controlled reproduction, larval culture, grow-out of juveniles to market size).

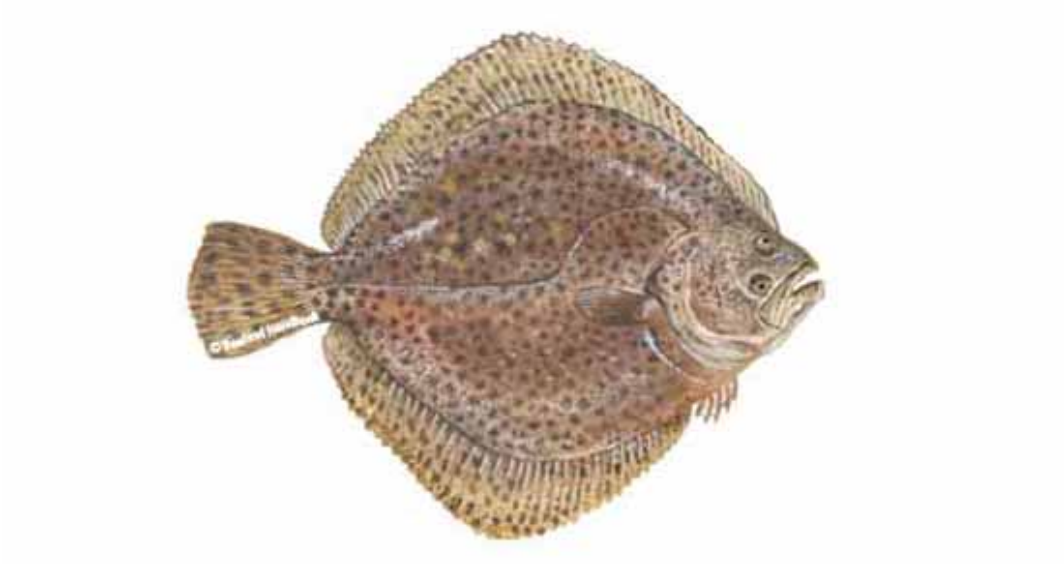

Figure 5. Turbot (*Scophthalmus maximus L.*). (Source: Seafoodsource, 2018).

At the beginning of the 1990's, technological development of juvenile production allowed to expand considerably aquaculture production of turbot and the number of farms. In Europe, turbot is cultured in Spain, France, and Portugal, but also in Denmark, Germany, Iceland, Ireland, Italy, Norway and Wales. European production has now stabilized around 9 000 to 11 000 tonnes per year (FEAP, 2017). Nowadays, fisheries

and aquaculture share almost equally the market. In Europe, Spain, France, Portugal, Iceland and Croatia are the top five producers' countries. In Portugal, turbot aquaculture production increased from 351 tons in 2008 to 2 222 tonnes in 2016, however, in 2012 the total production almost doubled that of 2016, with 4 500 tonnes (FEAP, 2017).

Turbot is a high trophic level species (4.4; FishBase) and it has high dietary protein requirement, even for a carnivorous fish (although protein retention is also very high), ranging from more than 50 to 65 % of the diet (Andersen and Alsted, 1993; Danielssen and Hjernes, 1993; Lee et al., 2003; Cho et al., 2005). Practical diets for turbot usually include high levels of FM (Fournier et al., 2004; Bonaldo et al., 2011). The high incorporation levels of FM have been recognized as one of the most important constraints to the sustainable development of turbot aquaculture production. Least- cost environmentally friendly formulations are therefore required for turbot to ensure the long-term sustainability of its production in aquaculture, with focus on the efficient and responsible use of feed resources. In this context, different dietary formulation strategies, involving the use of single or mixtures of different plant feedstuffs, have been researched. However, high dietary incorporation levels of plant feedstuffs in general lead to a decrease of turbot growth performance, feed intake, and feed efficiency, and may also induce intestinal disorders. Moreover, only protein-rich plant by-products have been considered as potential alternatives to FM in diets for turbot (Regost et al., 1999; Day and González, 2000; Burel et al., 2000; Bonaldo et al., 2011; Nagel et al., 2012).

Previous studies demonstrated that FM can be replaced up to 20% by corn gluten meal (Regost et al., 1999) or by 25% of soy protein concentrate (Day and González, 2000) without adverse effects on growth performance and feed efficiency. Bonaldo et al. (2015) also reported similar growth performance of turbot when dietary FM was reduced from 50% to 35% by the incorporation of plant protein mixture (wheat gluten, soybean meal, and soy protein concentrate). In general, SBM protein concentrate, and extruded lupin can substitute up to 25% fishmeal protein (Day and González, 2000; Burel et al., 2000), corn gluten meal up to 33% (Regost et al., 1999) and rapeseed protein isolate up to 33% (Nagel et al., 2012). A balanced mixture of plant feedstuffs may replace 20% of dietary protein (39% inclusion level) without negatively affect growth, digestibility, feed intake and feed efficiency or integrity of intestinal epithelium (Bonaldo et al., 2011).

1.5 Gilthead seabream

Gilthead seabream, *Sparus aurata* (Figure 6) is a common species in the Mediterranean Sea and Eastern Atlantic coasts. It can be found in marine and brackish water due to their euryhaline and eurythermal habits (FAO, 2014).

Figure 6. Gilthead Seabream (*Sparus aurata*). (Source: Fisheries-EU, 2018).

Gilthead seabream is mainly produced in intensive rearing systems being one of the most important marine species reared in Europe, particularly in the Mediterranean region (Oliva-Teles, 2000). Among the 137 different seafood species produced in European aquaculture in 2015, gilthead seabream is the third most produced fish, preceded by Atlantic salmon and rainbow trout (Eurostat, 2018). Total production of gilthead seabream has been increasing since 2000, even though with cyclic ups-and-downs. In 2016, the EU produced around 700 227 tonnes of gilthead seabream. Moreover, in 2016, compared to 2015, gilthead seabream consumption increased 6% (EUMOFA 2018). Greece is the largest producer in the EU (56 000 tonnes in 2016) and Portugal totalized 1 500 tonnes of gilthead seabream (EUMOFA, 2018; FEAP, 2017; INE 2017).

Gilthead seabream has a good market price, high growth and survival rate, being a species with great economic importance in Mediterranean aquaculture (Basurco et al., 2011; Oliva-Teles et al., 2011). It is adapted to different methods of aquaculture production such as recirculating aquaculture systems (RAS) and marine cages. Currently, RAS is mainly used for the first steps of aquaculture production (reproduction and growth phase), while grow out is performed in marine cages at open sea (Merinero et al. 2005).

Gilthead seabream is mainly carnivorous (trophic level of 3.7; FishBase), feeding on crustaceans and mollusks, as well as polychaetes, some teleost fish and

echinoderms, but can be accessorily herbivorous (Wassef and Eisawy 1985). These feeding habits are reflected in the higher protein requirement to achieve maximum growth. Protein requirement was estimated to be 45-46% for juveniles (Santinha et al., 1996; Vergara et al., 1996b), increasing up to 55% for fry (Vergara et al., 1996a). Dietary optimum lipid level was estimated to be 15-22% (Vergara and Jauncey, 1993; Vergara et al., 1996b; Santinha et al.,1999; Vergara et al., 1999).

FM is still an important feedstuff in gilthead sea bream aquafeeds, but FM and FO are being partially replaced, mainly by raw materials of plant origin. The use of plant protein sources in diets for gilthead seabream has been extensively studied (Robaina et al., 1995, 1997; Pereira and Oliva-Teles, 2002, 2003, 2004; Oliva-Teles., 2011; Monge-Ortiz et al., 2016;). SBM has been one of the most promising alternatives to FM and, according to Martinez-Llorens et al. (2007) the dietary soybean meal can be included in the diets up to 30% without affecting the growth or feed efficiency in gilthead seabream juveniles. Pereira and Oliva-Teles (2003) concluded that corn gluten meal, without amino acid supplementation, was found to successfully replace up to 60% of FM protein in diets for juveniles with no negative effects on fish performance, while 80% inclusion of corn gluten meal resulted in decreased growth and protein digestibility (Pereira and Oliva-Teles, 2003). However, in general, poor growth is observed in fish fed diets with high inclusion of plant protein sources due to deficiency of some EAA (Ye et al, 2011; Oliva-Teles., 2011). Moreover, other authors reported the successful 100% FM replacement by a mix of plant protein supplemented with EAA in diets for gilthead seabream juveniles (Kissil and Lupatsch, 2004). However, Sitja-Bobadilla et al. (2005) reported that total replacement of FM by a mixture of plant protein, supplemented with EAA, decreased growth performance and feed intake and increased liver fat deposition of sea bream juveniles (Sitja-Bobadilla et al., 2005). Several other plant protein ingredients have also been studied but, due to its nutritional characteristics, showed to have relatively lower inclusion potential, as sunflower meal and lupin meal (Robaina et al., 1997; Martínez-Shearer, 2000; Llorens et al., 2007).

1.6 Objectives

Valorization of alternative feedstuff for aquafeeds will require concentration of research efforts in order to take full advantage of the available alternatives and to account for all possible repercussions on growth, feed utilization, metabolism, and welfare of fish. Therefore, the present work aimed to evaluate the nutritional potential of corn-DDGS in marine aquaculture species with carnivorous habits. The biological models used were two marine fish species of interest for Mediterranean aquaculture: turbot (*Scophthalmus maximus*) and gilthead seabream (*Sparus aurata*).

Using a multi-disciplinary approach, this work aimed to evaluate DDGS use in aquafeeds, and the use of some additional strategies such as the use of exogenous enzyme or AA supplementations.

Specific aims included:

- Evaluate the effect of replacement of FM by DDGS in diets for turbot juveniles
- Evaluate the effect of exogenous enzymes supplementation of DDGS based diets for turbot juveniles
- Evaluate the effect of replacement of SBM by DDGS in diets for gilthead seabream juvenile.
- Evaluate the potential of dietary tryptophan supplementation of DDGS based diets to mitigate chronic stress in gilthead seabream

Chapter 2

Dietary replacement of fishmeal by corn distillers dried grains with solubles (DDGS) in diets for turbot (*Scophthalmus maximus*, Linneaus, 1758) juveniles

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ABSTRACT

The aim of this study was to determine the potential of corn distillers dried grain with solubles (DDGS) to partially replace fishmeal (FM) in practical diets for turbot. For that purpose, a control diet was formulated to include 40% FM and a mixture of plant protein ingredients (soybean meal, corn gluten, and wheat gluten). Three other diets were formulated based on the control but with 10, 17.5, or 25% of DDGS replacing FM. Diets were tested in triplicate, in an 84-days growth trial with juveniles of 29g initial body weight. Feed intake was not affected by diet composition, but growth and feed efficiency linearly decreased with the increase of dietary DDGS level. Whole-body dry matter and protein contents were not affected by diet composition, but lipid and energy content was higher in fish fed the control diet than the 17.5DDGS and 25DDGS diets and the 25DDGS diet, respectively. The apparent digestibility coefficients (ADCs) of protein and amino acids were similar among diets, while the ADCs of energy decreased with the increase of dietary DDGS level. Digestive amylase and lipase activities in the posterior intestine were lower in fish fed the 17.5DDGS and 25DDGS diets than the control diet, while proteases activity was not affected by diet. No differences among dietary treatments were observed on plasma glucose, but plasma total protein, albumin, triglycerides, and cholesterol were lower in fish fed the DDGS diets. Activity of key enzymes of glycolysis, gluconeogenesis, and lipogenesis was not affected by diet composition, but the activity of alanine aminotransferase increased with the increase of dietary DDGS. Moreover, the oxidative status of liver and intestine was not affected by dietary treatments, but susceptibility to oxidative stress was higher in the intestine than in the liver. Overall, it is concluded that replacing FM by DDGS in practical diets for turbot juvenile reduced growth performance and impaired overall nitrogen and energy metabolism.

Keywords: DDGS, digestibility, digestive enzymes, growth performance, nutrient utilization, oxidative stress, intermediary metabolism

1. Introduction

Fishmeal (FM) is becoming a scarce commodity in the world market, due to availability fluctuations and increasing global demand (Gatlin et al. 2007; Tacon and Metian, 2015). Given this context, various attempts have been made to reduce aquafeeds reliance on FM using alternative protein sources of good nutritional value in fish diets (Gatlin et al., 2007; Oliva-Teles et al. 2015). This is particularly critical for high trophic level species that are less flexible in terms of feed ingredient, and so more dependent upon the dietary use of FM and protein-rich plant ingredients than low-trophic level species (Tacon and Metian, 2015).

Turbot (*Scophthalmus maximus*) is an important aquaculture flatfish species in Europe and East Asia due to its high market acceptability, fast growth rate, and tolerance to intensive production conditions. Being a high trophic level species (4.4; FishBase), turbot has high dietary protein requirements, ranging from 50% to 65% (Andersen and Alsted, 1993; Danielssen and Hjernes, 1993; Lee et al., 2003; Cho et al., 2005). Although FM is traditionally used as the main protein source in turbot diets, previous studies demonstrated that it can be replaced up to 20% by corn gluten meal (Regost et al., 1999) or by 25% of soy protein concentrate (Day and González, 2000) without adverse effects on growth performance and feed efficiency. Bonaldo et al. (2015) also reported similar growth performances of turbot when dietary FM was reduced from 50% to 35% by the incorporation of plant proteins mixture (wheat gluten meal, soybean meal, and soy protein concentrate).

Though plant protein concentrates proved suitable feedstuffs to partially replace FM in turbot diets, they are expensive commodities, and search for alternative feed ingredients is required. Within these alternative ingredients are distillers dried grains with solubles (DDGS), which are by-products of cereal distillation for ethanol production, that are increasingly available at relatively low cost in the market (Pahm et al., 2008; Cozannet et al., 2011). DDGS is the most important by-product of the bioethanol manufacture industry, representing 0.3 tonnes per ton of processed cereal (Alagón et al., 2016). DDGS protein content ranges from 26 to 33%, fat from 9 to 14%, and neutral detergent fibres from 33 to 44% (Nyachoti et al., 2005; Kluth and Rodehutscord, 2010; Abdel-Raheem et al., 2011; Liu, 2012). Except for its fibre content, DDGS does not contain other anti-nutritional factors as those found in other plant protein sources such as soybean meal, rapeseed meal or cottonseed meal, and that usually interfere with fish performance and health (Gatlin et al., 2007; Krogdahl et al. 2010; Oliva-Teles et al.

2015). Moreover, DDGS is locally available, including in Europe, increasing its potential as an alternative to imported traditional plant ingredients.

DDGS has been mainly used in farm animal feeds (US Grains Council, 2012). Studies in fish evaluating FM replacement by DDGS are limited to a few fish species, such as in rainbow trout, *Oncorhynchus mykiss* (Cheng and Hardy, 2004), channel catfish, *Ictalurus punctatus* (Tidwell et al., 1990; Webster et al., 1991, 1992; Robinson and Li, 2008; Lim et al., 2009; Li et al., 2011), sunshine bass, *Morone chrysops* × *Morone saxatilis* (Thompson et al., 2008), hybrid tilapia, *Oreochromis niloticus* ♀×*Oreochromis aureus* ♂ (Welker et al., 2014a), meagre, *Argyrosomus regius,* and European seabass, *Dicentrarchus labrax* (Magalhães et al., 2015).

Thus, the aim of the present study was to evaluate the effect of partial replacement of FM by DDGS in diets for turbot juveniles on growth performance, diet digestibility, and some key actors involved in nutrient metabolism and oxidative defence mechanisms.

2. Materials and Methods

This experiment consisted of a growth trial and a digestibility trial performed with turbot (*Scophthalmus maximus*) juveniles provided by a commercial fish farm. The trials were conducted at the Marine Zoological Station, University of Porto, Portugal, by certified scientists (following the Federation of European Laboratory Animal Science Associations —FELASA category C recommendations) and the experiment was performed according to the European Economic Community animal experimentation guidelines directive of 24 November 1986 (86/609/EEC).

2.1 Experimental diets

A control diet was formulated to contain 40% FM (corresponding to 55% of dietary protein) and a mixture of plant ingredients (soybean meal, corn gluten, wheat gluten) as protein sources, and fish oil as lipid source (Table 1). Three other diets were formulated similar to the control but with FM partially replaced by 10%, 17.5%, or 25% of DDGS (corresponding to a FM protein replacement of circa 8, 14, and 20%, respectively). All dietary ingredients were finely ground, mixed, and dry pelleted using a laboratory pellet mill (CPM: California Pellet Mill, Crawfordsville, IN, USA) through a 3 mm dye, dried at 50°C for 24h, and then kept in a freezer until used.

Table 1. Composition and proximate analysis (% dry matter) of the experimental diets.

Diet	Control	10DDGS	17.5DDGS	25DDGS
Ingredients (% DM)				
Fish meal[1]	40.0	36.9	34.5	32.2
DDGS[2]	-	10	17.5	25
Wheat gluten[3]	5	5	5	5
Corn gluten[4]	15	15	15	15
Soybean meal[5]	10	10	10	10
Wheat meal[6]	16.8	10.3	5.5	0.7
Fish oil	9.7	9.3	9.0	8.6
Vitamin premix[7]	1.0	1.0	1.0	1.0
Choline chloride (50%)	0.5	0.5	0.5	0.5
Mineral premix[8]	1.0	1.0	1.0	1.0
Binder[9]	1.0	1.0	1.0	1.0
Proximate composition				
Dry matter (%)	88.5	86.5	85.8	86.3
Crude protein	53.5	54.4	54.4	53.7
Crude lipid	15.5	15.9	14.7	14.8
Ash	10.1	9.7	9.1	9.4
Gross energy (kJ g^{-1})	23.1	21.8	23.0	22.28
Essential amino acids content				
Lysine	3.52	3.46	3.31	3.28
Arginine	3.81	3.79	3.71	3.65
Histidine	1.86	1.88	1.87	1.89
Isoleucine	2.55	2.49	2.57	2.55
Leucine	4.91	5.19	5.51	5.86
Valine	2.94	2.83	2.88	2.91
Methionine	1.41	1.43	1.39	1.36
Phenylalanine	2.62	2.68	2.81	2.84
Threonine	2.1	2.16	2.33	2.38
Non-essential amino acids content				
Tyrosine	2.10	2.22	2.38	2.27
Aspartic Acid	4.20	4.25	3.88	3.86
Glutamic Acid	9.18	9.16	9.05	8.74
Serine	2.54	2.63	2.37	2.27
Glycine	2.63	2.53	2.53	2.48
Alanine	3.34	3.43	3.56	3.52
Proline	3.77	3.81	3.79	3.31

[1]Pesquera Centinela, Steam Dried LT, Chile (CP: 74.2%; CL 10.1%). Sorgal, S.A. Ovar, Portugal

[2]DDGS (CP: 32.8%; CL:9.0%; ash: 5.1%; crude fiber 7.5; acid detergent fiber: 14.4; neutral detergent fiber: 42.3; nitrogen free extract: 45.5; Pannonia Gold®)

[3]Wheat gluten (CP: 84.3%; CL: 3.9%), Sorgal, S.A. Ovar, Portugal

[4]Corn gluten (CP: 68.3%; CL: 2.9%), Sorgal, S.A. Ovar, Portugal

[5]Soybean meal (CP: 53.7%; CL:2.1%), Sorgal, S.A. Ovar, Portugal

[7]Vitamins (mg kg^{-1} diet): retinol, 18000 (IU kg^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.

[8]Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.4 (g kg^{-1} diet).

[9]Aquacube. Agil, UK.

2.2 Growth trial

The growth trial was performed in a thermo-regulated recirculating water system, equipped with a battery of 12 fiberglass cylindrical tanks (100L water capacity each), supplied with a continuous flow of filtered seawater (6L min^{-1}). During the trial, water temperature and salinity averaged 18 ±1°C and 35.5 ± 0.8 ‰, respectively. Oxygen levels were kept above 7.0 mg L^{-1} and a 12L:12D photoperiod was adopted.

For the growth trial, 12 homogenous groups of 18 fish each (initial body weight 30g) were constituted and each diet was randomly assigned to triplicate of these groups. The trial lasted 84 days and fish were fed by hand, twice a day, six days a week, to apparent visual satiety. Utmost care was taken to avoid feed waste and to assure that all feed supplied was consumed. Fish were bulk weighted at the beginning, after two weeks, and at the end of the trial, following 1 day of feed deprivation. Ten fish from the initial stock population and 6 from each experimental tank at the end of the trial were sampled, pooled, and frozen at -20°C for whole-body composition analysis. Whole fish, viscera and liver weights of these fish were recorded for determination of hepatosomatic (HSI) and visceral indices (VI).

At the end of the growth trial, fish continued to be fed for 3 more days to minimize manipulation stress, and then 3 fish per tank were randomly sampled 4 h after feeding. Blood was collected from the caudal vein with a heparinized syringe and centrifuged at 10,000 g for 10 min. Plasma aliquots were frozen at −80 °C until plasma analysis. Then, fish were immediately euthanized with a sharp blow to the head and eviscerated in an ice-cooled tray. The livers were collected to measure the activities of key intermediary metabolism and antioxidant enzymes. The intestine was excised, adherent adipose and connective tissues were carefully removed, and divided into anterior, mid, and posterior portions. The anterior intestine is the portion directly after the stomach and included the two pyloric caeca; mid and posterior portions were obtained by division of the remaining part in two identical parts. The individual pH of each intestine section was determined in situ using a pH meter (pH Eutech Instruments - Oakton, Singapore). The digestive enzymes activity was measured in the 3 intestinal portions, while the antioxidant enzymes activity was measured in the posterior intestine. Only fish with digestive contents throughout the intestinal tract were sampled. The liver and intestine portions were immediately frozen in liquid nitrogen and then stored at −80°C until use.

2.3 Digestibility trial

Fish remaining after the final sampling of the growth trial were transferred to a digestibility system, which consisted of a thermoregulated recirculating water system equipped with 12 tanks (55 L water capacity each) with faeces settling column connected to the outlet of each tank (Cho et al., 1982). During the trial, water-flow was about 4.5 L min^{-1} per tank, water temperature averaged 18±1°C, salinity averaged 35‰, and dissolved oxygen was kept near saturation. A new batch of each experimental diet was prepared adding to each diet 0.5% of chromium oxide as external marker. Triplicate homogenous groups of 15 fish with an initial body weight of 75g were randomly distributed to each tank and fed by hand twice a day, to apparent visual satiety. After 10 days of adaptation to the experimental diets and experimental conditions, faeces accumulated in each settling column were collected during 22 consecutive days, before the morning meal. Faeces were immediately centrifuged at 3 000 x g for 10 minutes, pooled for each tank, and stored at -20°C until analysis. One hour after the afternoon meal, tanks, pipes, and settling columns were thoroughly cleaned to remove faeces and uneaten feed.

Apparent digestibility coefficients (ADC) of dry matter, protein, energy, and amino acids of the experimental diets were calculated as follows:

$$\mathrm{ADC_{diet}} = \left(1 - \left(\frac{\text{dietary } \mathrm{Cr_2O_3} \text{ level} \times \text{faeces nutrient or energy level}}{(\text{faeces } \mathrm{Cr_2O_3} \text{ level} \times \text{dietary nutrient or energy level})}\right)\right) \times 100$$

2.4 Analytical methods

2.4.1 Chemical analysis

Chemical analysis of ingredients, diets, and faeces were conducted as follows: dry matter by drying the samples at 105°C until constant weight; protein content (N x 6.25) by the Kjeldahl method following acid digestion, Kjeltec digestion using and distillation units (Tecator Systems, Höganäs, Sweden; models 1015 and 1026, respectively); lipid content by extraction with petroleum ether using a Soxtec system (Tecator Systems, Höganäs, Sweden; extraction unit model 1043 and service unit model 1046); ash by incineration in a muffle furnace at 450 °C for 16 h; gross energy by direct combustion of samples in an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261); chromic oxide by acid digestion, according to Furukawa and Tsukahara (1966).

Amino acid content of the experimental diets and faeces were analysed, after hydrolysis with 6 N hydrochloric acid (at 112 °C; under N2 atmosphere; for 23 h), in a

high-pressure liquid chromatography (HPLC) using a Pico-Tag Amino Acid Analysis System (Waters auto sample model 717 plus; Waters binary pump model 1525; Waters dual absorbance detector model 2487; Waters, USA), according to the procedures described by Banuelos-Vargas et al. (2014). Tryptophan and cysteine were not measured.

Hepatic glycogen content was determined by amyloglucosidase hydrolysis, following the method described by Roehrig and Allred (1974) and hepatic lipids were determined gravimetrically according to Folch et al. (1957).

2.4.2 Plasma analysis

Plasma metabolites were analysed using commercial kits from Spinreact: glucose (ref: 1001191), total protein (ref: 1001291), albumin (ref: 1001020), triglycerides (TAG; ref: 1001312), and total cholesterol (ref: 1001090).

2.5 Enzyme activity

Liver and intestine sections were homogenized in ice-cold buffer (100mM-Tris-HCl, 0.1mM-EDTA and 0.1 % triton X-100 (v/v), pH 7.8) and centrifuged at 30 000g for 30 min at 4 °C. The resultant supernatants were collected and aliquots were stored at -80°C until enzyme activity analysis. All enzyme activities were measured at 37°C in a microplate reader (ELx808™; BioTek Instruments), by monitoring changes in absorbance.

2.5.1 Digestive enzymes activities

α-Amylase (EC 3.2.1.1) activity was determined with a commercial kit (ref. 41201, Spinreact, Girona, Spain); the rate of product formation (2-chloro-4-nitrophenol) was quantified at 405 nm. Lipase (EC 3.1.1.3) activity was determined using a commercial kit (ref. 1001275, Spinreact, Girona, Spain); 1-2-O-dilauryl- rac-glycero-3-glutaric acid- 60-methylresorufin-ester was used as substrate, and the formation rate of methylresorufin was followed at 580 nm. Total protease activity was measured by the casein-hydrolysis method. A reaction mixture containing casein at 1% (w/v), buffer (0.1 M Tris HCl at pH 7.8), and supernatant from the homogenates was incubated for 1 h at 37°C, and the reaction stopped by adding trichloroacetic acid solution (8%; w/v). Then, the reaction mixture was kept for 1 h at 2°C, centrifuged at 1800 g for 10 min and the supernatant absorbance measured at 280 nm against blanks. A control blank for each sample was prepared adding the supernatant from the homogenates after incubation. Tyrosine solution was used as standard. One unit (U) of enzyme activity was defined as μmol of product generated per minute under the measurement conditions described above and

expressed per mg soluble protein (specific activity). Protein concentration was determined using Bradford's method (1976), with bovine serum albumin solution as standard.

2.5.2 Intermediary metabolism enzymes activities

Hexokinase (HK; EC 2.7.1.1) and glucokinase (GK; EC 2.7.1.2) activities were determined as described by Vijayan et al. (1990), and the reaction mixture contained 50 mM imidazole-HCl buffer (pH 7.4), 2.5mMATP, 5mMMgCl2, 0.4mMNADP, 2 units mL^{-1} G6PDH and 1 mM (HK) or 100 mM (HK-IV) glucose. Pyruvate kinase (PK; EC 2.7.1.40) activity was performed with a reaction mixture consisting of 50 mM imidazole-HCl buffer (pH 7.4), 5 mM MgCl2, 100 mM KCl, 0.15 mM NADH, 1 mM ADP, 2 units mL^{-1} LDH and 2 mM PEP (Morales et al., 1990). Fructose 1,6-bisphosphatase (FBPase; EC 3.1.3.11) activity was performed with a reaction mixture consisting of 50 mM imidazole-HCl buffer (pH 7.4), 5 mM MgCl2, 12 mM 2-mercaptoethanol, 0.5 mM NADP, 2 units mL^{-1} G6PDH, 2 units mL^{-1} PGI and 0.5mMfructose 1,6- bisphosphate (Morales et al., 1990). Glutamate dehydrogenase (GDH; EC 1.4.1.2) activity was performed using a reaction mixture containing 50 mM imidazole–HCl buffer (pH 7.4), 0.2 mM NADH, 1 mM ADP, 100 mM ammonium acetate, 2 units mL^{-1} LDH and 10 mM α-ketoglutarate (Morales et al., 1990). Aspartate aminotransferase (ASAT; EC 2.6.1.1) activity was determined as previously described by Singer et al. (1990) and the reaction mixture contained 50 mM imidazole-HCl buffer (pH 7.4), 10 mM α-ketoglutarate, 0.3 mM NADH, 0.05 mM pyridoxal phosphate, 3 units mL^{-1} MDH and 25 mM L-aspartate. Alanine aminotransferase (ALAT; EC 2.6.1.2) activity was determined as described by Morales et al. (1990) and the reaction mixture contained 50 mM imidazole-HCl buffer (pH 7.4), 10 mM α-ketoglutarate, 0.2 mM NADH, 0.05 mM pyridoxal phosphate, 2 units mL^{-1} LDH and 25 mM L-alanine. Glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity was measured as described by Morales et al. (1990), using a reaction mixture containing 50 mM imidazole-HCl buffer (pH 7.4), 5 mM MgCl2, 2 mM NADP and 1 mM glucose-6-phosphate. All enzyme activities are expressed as milliunits per milligram of soluble protein (specific activity).

2.5.3 Antioxidant enzymes activities

Superoxide Dismutase (SOD; EC 1.15.1.1) activity was measured at 550 nm by the ferricytochrome C method, using xanthine/xanthine oxidase as the source of superoxide radicals (McCord and Fridovich, 1969). The reaction mixture consisted of 50 mM-potassium phosphate buffer (pH 7.8 Sigma), 0.1 mM-EDTA (Sigma), 0.1 mM-xanthine (Sigma), 0·012 mM-cytochrome C (Sigma) and 0.025 IU/mL xanthine oxidase

(Sigma). SOD activity is expressed as units per mg of protein. One unit of activity is defined as the amount of enzyme necessary to produce 50% inhibition of ferricytochrome C reduction rate. Catalase (CAT; EC 1.11.1.6) activity was determined according to Aebi (1984) by measuring the decrease in H_2O_2 concentration at 240 nm. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7, Sigma) and 10 mM-H2O2 (Sigma) freshly added. Glutathione peroxidase (GPX; EC 1.11.1.9) activity was assayed as described by Flohé and Günzler (1984). The oxidized glutathione (GSSG) generated by GPX was reduced by glutathione reductase (GR), and NADPH consumption rate was monitored at 340 nm. The reaction mixture consisted of 50 mM-potassium phosphate buffer (pH 7.1, Sigma), 1 mM-EDTA, 3.9 mM-GSH, 3.9 mM-sodium azide (Sigma), 1 IU/ml GR (Sigma), 0.2 mM-NADPH (Sigma) and 0.05 mMH_2O_2 (Sigma). Glutathione reductase (GR; EC 1.6.4.2) activity was determined at 340 nm by measuring the oxidation of NADPH as described by Morales et al. (2004). The reaction mixture consisted of 0.1 M-sodium phosphate buffer (pH 7.5, Sigma), 1 mM-EDTA, 0.63 mM-NADPH (Sigma) and 0.16 mM-GSSG (Sigma). Except for SOD, whose units of expression were described above, all other enzyme activities were expressed as units (CAT) or milliunits (GPX and GR) per mg of soluble protein. One unit of enzyme activity was defined as the amount of enzyme required to transform 1 µmol of substrate/min under the above assay conditions.

2.6 Lipid peroxidation

Lipid peroxidation levels were determined based on the concentration of malondialdehyde (MDA) following the methodology described by Buege and Aust (1978). An aliquot of supernatant from the homogenate (100 µL) was mixed with 500 µL of a previously prepared solution containing 15% (w/v) TCA (Sigma), 0.375% (w/v) thiobarbituric acid (Sigma), 80% (v/v) HCl 0.25 N and 0·01% (w/v) butylated hydroxytoluene (Sigma). The mixture was heated to 100 °C for 15 min. After being cooled to room temperature and centrifuged at 1500g for 10 min, the absorbance was measured at 535 nm in the supernatant. MDA concentration was expressed as nmol MDA per g of wet tissue, calculated from a calibration curve.

2.7 Statistical analysis

Data were checked for normality and homoscedasticity and if needed normalized (log normalization or arcsine square root transformation). The effect of the dietary treatments on the different parameters was analysed by one-way ANOVA. Significant differences among means ($p<0.05$) were determined by the Tukey's multiple range test.

All statistical analyses were carried out using the SPSS version 22.0 software package for Mac.

3. Results

Fish promptly accepted all diets, and feed intake (g kg $ABW^{-1}day^{-}1$) was not affected by diet composition (Table 2). Mortality was very low during the trial, and not affected by dietary treatments. At the end of the growth trial, fish performance, evaluated by final body weight, weight gain, and daily growth index, linearly decreased with the increase of dietary DDGS levels. Feed efficiency (FE), protein efficiency ratio (PER), nitrogen retention (NR) and energy retention (ER) (% digestible nitrogen or energy

Table 2. Growth performance, nutrient retention, whole-body and liver composition of turbot fed the experimental diets.

Diets	Initial	Control	10DDGS	17.5DDGS	25DDGS	SEM	Linear regression *p*-value	R^2
IBW (g $fish^{-1}$)	–	29.5	29.5	29.5	29.5	0.01		
FBW (g $fish^{-1}$)	–	87.9^{b}	76.5^{ab}	66.3^{a}	64.4^{a}	2.76	0.00	0.79
WG (g kg $ABW^{-1}day^{-1}$)[1]	–	11.8^{c}	10.6^{bc}	9.2^{ab}	8.8^{a}	0.33	0.00	0.81
DGI[2]	–	1.6^{b}	1.4^{ab}	1.1^{a}	1.1^{a}	0.06	0.00	0.81
FI (g kg$ABW^{-1}day^{-1}$)[3]	–	9.3	10.3	9.6	9.6	0.20	0.81	0.01
FE[4]	–	1.3^{b}	1.0^{ab}	1.0^{a}	0.8^{a}	0.05	0.00	0.59
PER[5]	–	2.4^{b}	1.9^{ab}	1.8^{a}	1.6^{a}	0.91	0.00	0.87
NR (% DNI)[6]	–	45.8	37.5	31.5	31.4	2.29	0.01	0.54
ER (% DEI)[7]	–	43.0^{c}	39.2^{bc}	36.4^{b}	31.1^{a}	1.60	0.01	0.50
Mortality (%)	–	0.0	0.0	3.7	0.0	0.62		
HSI[8]	–	1.3	1.2	1.1	1.2	0.29		
VI[9]	–	6.2	6.2	6.3	6.3	0.87		
Whole-body composition (% wet weight)								
Dry matter (%)	22.5	24.2	24.7	23.6	24.01	0.17		
Protein	15.1	15.9	16.2	15.5	15.8	0.24		
Lipids	2.6	5.1^{b}	5.4^{b}	3.9^{a}	3.9^{a}	0.21		
Ash	5.1	4.7	4.2	4.9	5.0	0.15		
Energy (kJ g^{-1} DM)	4.4	5.4^{b}	5.3^{ab}	5.0^{ab}	4.8^{a}	0.08		
Liver composition (% wet weight)								
Liver Glycogen	–	24.2	13.4	12.7	11.7	1.88		
Liver Lipids	–	16.0^{ab}	14.9^{ab}	12.8^{a}	17.6^{b}	0.80		

Values presented as means (n = 3) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters are significantly different ($p < 0.05$).
ABW: average body weight = ((initial body weight, IBW + final body weight, FBW)/2)
[1]Weight gain = ((FBW-IWB) × 1000) / (ABW × time in days).
[2]Daily growth index = ($(FBW^{1/3} – IBW^{1/3})$/ time in days) × 100.
[3]Feed intake = (total dry feed intake × 1000) / (ABW × time in days).
[4]Fed efficiency = wet weight gain / dry feed intake.
[5]Protein efficiency ratio = wet weight gain / crude protein intake.
[6]Nitroegn retention (%DNI) = whole-body nitrogen retention/ digestibility nitrogen intake; NDI) × 100.
[7]Energy retention (%DEI) = whole-body energy retention/ digestibility nitrogen intake; EDI) × 100.
[8]Hepatosomatic index: (liver weight/body weight) × 100.
[9]Visceral index: (viscera weight/body weight) × 100.

intake) were also not affected by dietary treatments. HSI and VI were not affected by dietary treatments. At the end of the growth trial no differences in whole-body dry matter, protein, and ash content among groups were observed. Whole-body lipid content was

higher in fish fed with the control and 10DDGS diets than with the 17.5DDGS and 25DDGS diets, and energy content linearly decreased with the increase of DDGS in the diets. Though ANOVA indicated that there is a significant difference in glycogen content, Tukey was not able to discriminate. Liver lipids were higher with the 25DDGS diet than 17.5DDGS diet.

The apparent digestibility coefficients (ADCs of dry matter ranged between 61 and 67%, the ADCs of protein ranged between 85 and 87% and the ADCs of amino acids ranged between 80 and 88%), were not affected by diet composition. The ADCs of energy ranged between 66 and 76%, and decreased with the increase of DDGS in the diets (Table 3).

Table 3. Apparent digestibility coefficients (ADCs %) of nutrients and energy of the experimental diets in turbot.[1]

Diets	Control	10DDGS	17.5DDGS	25DDGS	SEM
Dry matter	67.2	61.7	61.6	61.1	1.7
Protein	86.7	85.2	85.0	86.4	0.5
Energy	76.2^{b}	70.0^{ab}	68.8^{ab}	65.7^{a}	1.6
Essential amino acids					
Lysine	83.3	84.2	83.3	81.2	0.68
Arginine	83.6	83.0	85.0	80.7	0.68
Histidine	85.0	84.9	83.2	81.2	0.94
Isoleucine	85.1	85.9	84.8	79.8	0.99
Leucine	84.1	87.4	87.0	88.0	0.67
Valine	81.5	84.8	84.8	83.7	0.64
Methionine	84.8	83.6	83.6	81.5	0.57
Phenylalanine	83.0	84.3	84.3	81.3	0.55
Threonine	83.7	84.6	84.2	80.7	0.74
Non-essential amino acids					
Tyrosine	81.2	83.0	83.5	82.1	0.51
Aspartic Acid	81.3	85.0	84.1	81.3	0.79
Glutamic Acid	85.2	84.7	86.1	88.1	0.63
Serine	84.9	85.6	85.2	80.0	0.95
Glycine	83.4	86.9	85.6	83.3	0.76
Alanine	86.7	85.1	85.7	83.3	0.78
Proline	83.0	85.2	85.5	81.9	0.75

Values presented as means (n = 3) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters are significantly different ($P < 0.05$).

The intestine pH increased from anterior to posterior intestine (Table 4). In the anterior and mid intestine, pH values were higher in the DDGS groups than in the control, but no differences between groups were observed in the posterior intestine. Amylase activity was not affected by intestinal region, but it was lower in the posterior intestine of fish fed diets 17.5DDGS and 25DDGS than in the control. Sum of amylase activity was higher in the control fed fish than those fed the DDGS diets. Lipase activity increased along the intestine in the control group, but differences between intestine regions were

not significant in the DDGS levels. Sum of lipase activity decreased with dietary treatments. Protease activity increased along the intestine in all groups, but differences were not statistically significant in fish fed the 17.5DDGS diet. Sum of protease activity was not affected by diet composition.

Table 4. pH and specific activities (mU mg protein^{-1}) of amylase, lipase and proteases in the anterior, mid and posterior intestine of turbot juveniles fed experimental diets.

Diets	Control	10DDGS	17.5DDGS	25DDGS	SEM
pH					
Anterior	7.2^{aA}	7.6^{bA}	7.6^{bA}	7.4^{abA}	0.06
Mid	7.4^{aA}	7.8^{abAB}	7.7^{abAB}	7.9^{bB}	0.06
Posterior	7.9^{B}	8.0^{B}	7.9^{B}	8.0^{B}	0.03
Amylase					
Anterior	9.1	8.7	7.1	6.8	0.46
Mid	10.0	7.6	7.6	7.5	0.56
Posterior	9.4^{b}	7.3^{ab}	5.1^{a}	5.2^{a}	0.63
SUM	28.4^{b}	23.6^{ab}	19.7^{a}	19.5^{a}	1.12
Lipase					
Anterior	3.9^{A}	3.9	3.8	3.8	0.24
Mid	6.2^{AB}	5.9	5.1	4.5	0.49
Posterior	7.8^{bB}	7.0^{ab}	3.7^{a}	3.1^{a}	0.49
SUM	17.9^{c}	16.8^{b}	12.6^{a}	11.5^{a}	1.08
Protease					
Anterior	160.0^{A}	161.7^{A}	161.4	166.5^{A}	10.26
Mid	244.9^{A}	261.1^{B}	276.8	294.2^{B}	16.09
Posterior	399.4^{B}	366.7^{C}	266.8	256.5^{B}	21.83
SUM	804.3	789.5	705.0	717.2	27.22

Values presented as means (n = 9) and pooled standard error of the mean (SEM). Within a row, different small letters indicate differences (P<0.05) between dietary treatments; within a column, capital letters indicate differences (P<0.05) between intestinal sections.

Plasma glucose was not affected by dietary treatments (Table 5). While plasma total protein, albumin, and total cholesterol were higher in the control group than in the DDGS groups. Plasma triglycerides of the control group were higher than that of 10DDGS group.

Table 5. Plasma metabolites concentration (mg dL^{-1}) of turbot juveniles fed the experimental diets.

Diets	Control	10DDGS	17.5DDGS	25DDGS	SEM
Glucose	36.0	35.6	37.1	37.0	1.00
Total Protein (g dL^{-1})	3.4^{b}	3.0^{a}	3.0^{a}	3.0^{a}	0.64
Albumin	1.5^{b}	1.1^{a}	1.0^{a}	1.0^{a}	0.05
Triglycerides	207.5^{b}	163.3^{a}	183.1^{ab}	182.5^{ab}	5.15
Total cholesterol	100.6^{b}	80.6^{a}	76.0^{a}	73.1^{a}	2.82

Values presented as means (n = 9) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters are significantly different (P < 0.05).

Specific activity of key enzymes of glycolysis, gluconeogenesis and lipogenesis were not affected by the treatments (Table 6). The key enzymes for amino acid were also unaffected by diet composition, being significant for the alanine aminotransferase.

Table 6. Specific activities (mU mg^{-1} protein) of hepatic hexokinase (HK), glucokinase (GK), pyruvate kinase (HK), fructose-1,6-bisphosphatase (FBPase), glucose-6-phosphate dehydrogenase (G6PDH), glutamate dehydrogenase (GDH), alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT), in turbot juveniles fed the experimental diets.

Diets	Control	10DDGS	17.5DDGS	25DDGS	SEM
Glycolysis					
HK	3.09	2.81	2.87	2.64	0.09
GK	2.66	2.53	2.53	2.46	0.09
PK	1.80	1.81	2.02	2.01	0.56
Gluconeogenesis					
FBPase	11.1	10.8	10.8	10.5	0.26
Lipogenesis					
G6PDH	91.3	87.4	85.7	78.7	2.25
Amino acid catabolism					
GDH	268.7	276.2	282.1	292.2	6.95
ALAT	184.4^{a}	230.3^{ab}	264.3^{b}	272.3^{b}	10.7
ASAT	401.7	421.0	451.7	466.2	11.2

Values presented as means (n = 9) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters are significantly different ($P < 0.05$).

Specific activity of oxidative stress-related enzymes and lipid peroxidation values in the liver and posterior intestine were also not affected by the experimental diets (Table 7). Both oxidative stress enzymes activity and lipid peroxidation values were higher in the posterior intestine than in the liver.

Table 7. Specific activities of liver glutathione peroxidase (GPX), glutathione reductase (GR) (mU/mg protein), catalase (CAT), superoxide dismutase (SOD) (U/mg protein) and lipid peroxidation (LPO; nmol MDA g^{-1} tissue) in liver and posterior intestine of turbot fed the experimental diets.

Diets	Control	10DDGS	17.5DDGS	25DDGS	SEM
Liver					
GPX	2.7	1.9	2.0	1.8	0.16
GR	4.0	3.7	3.8	3.1	0.14
CAT	58.5	58.1	63.9	60.2	1.65
SOD	166.8	160.4	184.8	161.0	6.56
LPO	7.0	7.7	7.5	7.6	0.39
Posterior intestine					
GPX	26.8	29.4	26.5	27.5	1.52
GR	23.7	24.1	20.7	22.4	0.85
CAT	788.8	754.4	648.3	660.4	39.44
SOD	1101.2	1127.4	1077.3	1011.5	79.43
LPO	28.6	27.4	27.6	27.2	0.61

Values presented as means (n = 9) and pooled standard error of the mean (SEM). Absence of superscript indicates no significant difference between treatments.

4. Discussion

Studies on the potential of alternative feedstuffs to FM in turbot diets are centered on few plant feedstuffs, such as corn gluten meal (Regost et al. 1999), extruded lupin, heat-treated rapeseed meal (Burel et al. 2000), soybean protein concentrate (Day and Gonzalez 2000), and plant protein mixtures (wheat gluten meal, soy protein concentrate and soybean meal; Fournier et al. 2004; Bonaldo et al., 2011, 2015). However, new ingredients outside the human food chain should be taken into consideration to ensure an environmentally, economically, and energy friendly aquaculture production (Olsen, 2011). DDGS has been tested as a potential feedstuff for aquafeeds, particularly for omnivorous species, due to their higher tolerance to high dietary fibre content than carnivorous species. For instance, in Nile tilapia, *Oreochromis niloticus*, DDGS was successfully incorporated up to 50-55% in diets devoid of FM (Wu et al., 1996, 1997; Abo-State et al. 2009) and up to 60% if duly supplemented with lysine (Shelby et al., 2008). For hybrid tilapia lower DDGS levels were proposed, around 30% in replacement of one-third of dietary soybean meal (Welker et al., 2014a). Also, for yellow perch, *Perca flavescens* and milkfish, *Chanos*, DDGS may be included in the diet up to 40 and 45%, respectively, partially replacing soybean meal (Schaeffer et al., 2011; Mamauag et al., 2017). For channel catfish, DDGS may be incorporated in the diets up to 40% (Tidwell et al., 1990; Webster et al., 1992), up to 70% if the diets are supplemented with lysine (Webster et al., 1991), or up to 90% if used in winter diets (Webster and Tidwell, 1992). However, recent studies with channel catfish recommended lower dietary incorporation of DDGS, up to 20 or 30% in non-supplemented or lysine supplemented diets, respectively (Robinson and Li, 2008; Lim et al. 2009; Zhou et al. 2010; Renukdas et al., 2014). Also in hybrid striped bass, *Morone chrysops* × *M. saxatilis,* dietary inclusion of 33% DDGS did not affect fish performance, feed efficiency, fillet colour or consumer acceptance (Trushenski and Gause, 2013).

For high trophic level species lower dietary DDGS levels has been reported. For instance, in olive flounder (*Paralichthys olivaceus*) maximum dietary DDGS level that assured appropriated growth and feed efficiency was circa 14% (Rahman et al., 2015). In rainbow trout, DDGS may be incorporated at 15 and 22.5% in the diets replacing 50 or 75% of fish meal, provided that diets are supplemented with lysine and methionine (Cheng and Hardy, 2004). Also in rainbow trout, replacement of 50% of a plant feedstuffs mixture (sunflower meal, rapeseed meal, and field peas) by DDGS improved feed intake, weight gain, and feed utilization (Øverland et al., 2013). However, in other studies with rainbow trout it was observed that DDGS should be included in the diets only up to 10%,

even if supplemented with essential amino acids or phytase (Stone et al., 2005; Barnes et al., 2012).

Results of the present study also indicate that DDGS was not well utilized by turbot juveniles and that even a dietary inclusion of 10%, replacing 8% of FM protein, negatively affected growth performance. In general, FM replacement by alternative feedstuffs in turbot diets is successful just at low replacement levels. For instance, reduced turbot performance was also observed with the dietary replacement of 15% FM by rapeseed protein isolate (Nagel et al., 2012) or soybean meal (Xu et al., 2016). However, in other studies it was possible to replace 20% FM with corn gluten (Regost et al., 1999) or 25% FM with soy protein concentrate (Day and Gonzáles, 2000).

Levels of FM replacement by alternative feedstuffs in turbot diets seem to be dependent of FM level in the control diet. For instance, Bonaldo et al. (2011) concluded that by replacing 10% of FM by a mixture of plant feedstuffs in a diet with 55% FM just slightly, but not significantly, reduced growth performance. However, replacing 15% of FM by a mixture of plant feedstuffs in a diet with 50% FM significantly reduced growth and feed utilization (Bonaldo et al., 2015). Similarly, in the present study the control diet only included 40% FM, and reducing it to just 36.9% already affected turbot performance. Accordingly, Dong et al. (2016) also observed that dietary FM reduction from 62 to 40% by replacement with a mixture of plant and animal ingredients did not reduced turbot growth performance, while dietary FM reduction to 31% significantly affected fish performance. As highlighted by Øverland et al. (2013), differences in FM quality, FM dietary replacement level, and alternative feedstuff nutrient content and digestibility may greatly affect growth performance response, justifying the differences between present and previous studies on alternative feed ingredients for FM in turbot.

Palatability may be also an issue with dietary FM replacement, as alternative feedstuffs may not be so palatable and therefore voluntary feed intake may compromise, which in turn may affect growth performance. In the present study, although total FI per fish was lower in fish fed the DDGS-rich diets, per unit body weight basis (g kg $ABW^{-1}day^{-1}$), not differences were observed between diets. Dietary DDGS inclusion generally does not affect feed intake by fish (Herath et al., 2016; Mamauag et al., 2017). Inclusively, in rainbow trout it was observed that replacement of 50% of a mixture of plant feedstuffs (sunflower meal, rapeseed meal, and field peas) by DDGS increased feed intake (Øverland et al., 2013).

Since dietary inclusion of DDGS seemed to have impacted less on FI than on growth, differences in growth performance, feed efficiency, and protein efficiency ratio of

turbot fed diets with increasing DDGS levels may have resulted of poorer diet digestibility or metabolic utilization. Indeed, dietary inclusion of DDGS reduced dry matter and energy digestibility, probably due to the high indigestible non-starch polysaccharides (NSP) content of DDGS. For high trophic level species, digestibility of dry matter and energy is generally negatively correlated with the non-starch contents of feedstuffs (Tibbetts et al., 2006; Nagel et al., 2012; Magalhães et al., 2015), which may also impair digestibility of other nutrients (Sinha et al., 2011). However, in present study, protein and amino acid digestibility were not affected by dietary DDGS inclusion, confirming that DDGS protein and amino acids are highly digestible, as previously reported by other studies (Øverland et al, 2013; Welker et al., 2014b; Magalhães et al., 2015). Accordingly, for seabass, meagre, and milkfish, DDGS digestibility was high for protein (91- 98%) and lipids (82-89%), and moderate for energy (58-68%) and dry matter (52-57%). The lower ADCs of energy and dry matter may be related to the absence of enzymes required to digest NSP in fish (Sinha et al., 2011).

In the present study, irrespective of diet composition, intestinal pH increased along the intestine, as expected (Krogdahl et al., 2015). Intestinal pH within the range of values 2 to 8.0, being within the value previously observed in turbot (Diógenes et al., 2017) as well as in other fish species (Krogdahl et al., 2015). Chyme pH in the anterior and mid intestinal portion increased with FM replacement by DDGS. Chyme alkalization was also observed in seabream, but not in seabass, when fed carob seed germ meal based diets compared to FM based diets (Nikolopoulou et al., 2011). According to Krogdahl et al. (2015), a shift in intestinal transport of amino acid, some of which are H+ symporters and others are H+ exchangers, may affect the intestinal pH. Thus, these differences in chime pH may reflect the different amino acid composition of the diets and different rates of protein digestion along the intestinal tract.

Dietary inclusion of DDGS affected pancreatic enzymes activities, reducing amylase and lipase, but not protease activities. This is in accordance with the reduced ADC of energy in the diets including DDGS, and may be related to the increased NSP levels in DDGS diets, which are known to reduce intestinal trypsin and amylase activities in turbot (Hu et al., 2015). Also for other species, such as trout and seabass, FM replacement by plant proteins reduced digestive enzymes activities due to the presence of anti-nutritional factors in the diets, like anti-trypsin factors and NSPs (Santiogosa et al., 2008). However, such effects seem to be species-specific, and related to the trophic level of the species. For instance, in white seabream (*Diplodus sargus*), an omnivorous species, the inclusion of dietary plant feedstuffs providing up to 96.3% of total dietary protein did not affect pancreatic enzymes activities (Magalhães et al., 2016). In the

present study, lipase and proteases activities, but not amylase, increased along the intestine irrespective of treatments. This is in agreement with other studies in different species (Izquierdo and Henderson, 1998; Pérez-Jiménez et al., 2009; Magalhães et al., 2015, 2017; Diógenes et al., 2017) but in contradiction with the results of studies by Krogdahl et al. (2015) and Castro et al. (2013). Though chyme drag along the intestine and consequently the displacement of maximum enzyme activity to distal intestine cannot be discarded, the higher activity of enzymes in the posterior intestine may also reflect its higher role in the digestion process, as suggested by Izquierdo and Henderson (1998).

Even though protein and amino acid digestibility were not affected by the dietary inclusion of DDGS, there was a linear decrease of N retention (% digestible N intake) and of protein efficiency ratio, accompanied by a trend for an increase of amino acid catabolic enzymes activities and for a reduction of plasma protein and albumin levels. Together, these parameters point out to a reduction of digestible protein utilization efficiency. Previously, it was also reported that FM replacement by plant proteins might impair protein efficiency ratio and protein retention in turbot (Regost et al., 1999; Nagel et al., 2012; Bonaldo et al., 2011, 2015). Although the EAA content (% DP) of all diets fulfilled the estimated EAA requirements for turbot juveniles (Peres and Oliva-Teles, 2008), the digestible A/E ratio of the diets was affected by dietary DDGS level and this might, in part, explain the lower metabolic utilization efficiency of protein observed in the present study. Indeed, it was previously observed that not only total EAA content but also EAA profile might significantly affect protein utilization efficiency in fish (Peres and Oliva-Teles, 2007). The reduced protein retention in turbot fed DDGS diets might be also related to differences in muscle influx of free amino acids, decreased rapamycin (TOR) signalling activities, or amino acid response (AAR) signalling activities, as previously observed in turbot fed diets including soybean meal replacing FM (Xu et al., 2016). The reduction of plasma total protein and albumin also reflects the inefficient protein utilization in fish fed DDGS diets. Indeed, levels of plasma proteins, including total protein, albumin, and transferrin have been proposed as markers of protein malnutrition in turbot (Nagel et al., 2012) as well as in other fish species (Peres et al., 2013, 2014). This protein malnutrition may have also impaired the export of triglycerides and other lipids from the liver, resulting in lower plasma triglycerides and cholesterol as well as lipid accumulation in the liver, as it was observed in fish fed the 25DDGS diet. Dietary replacement of FM by alternative protein sources was also reported as modulating plasma triglycerides and cholesterol levels (Peres et al., 2003; Sitjá-Bobadilla et al., 2005).

Dietary replacement of FM by DDGS also decreased energy digestibility, which results in lower digestible energy intake. It has been pointed out that one of the major constrains of the use of DDGS in carnivorous fish diets is its high level of NSPs that reduce dry matter and energy digestibility and impairs energy metabolism (Welker et al., 2014b; Magalhães et al., 2015). The lower digestible energy intake in the DDGS groups was further reflected on lower growth performance, energy retention, and whole-body lipids and energy contents. Previous studies have also shown that moderate to severe restrictions of energy intake resulted in decreased energy retention efficiency and whole-body lipid content in turbot (Dietz et al., 2012) as well as in other fish species (Lupatsch et al., 2003; Peres and Oliva-Teles, 2005, 2011).

Despite the reduction of digestible energy intake, the activities of glycolytic and gluconeogenic enzymes were not affected, being in line with the unaffected plasma glucose levels. There was a trend for a decrease of G6PDH activity with the increase of DDGS in the diets. As this enzyme is responsible for NADPH production required for lipogenesis, this trend is consistent with the decrease of whole-body lipids of fish fed the DDGS diets. Similarly, Xu et al. (2016) found that expressions of genes involved in glycolysis and lipogenesis were suppressed in turbot fed soybean meal-based diets compared to FM based diets, indicating that this the cause of the lower lipid retention.

Fish, as other vertebrates, have enzymatic and non-enzymatic mechanisms that help to prevent the oxidative damages of reactive oxygen species (ROS), produced by the reduction of molecular oxygen within biological systems. A disturbance of the balance between antioxidant capacity and ROS production induce oxidative stress, damaging cell structures including nucleic acids, lipids, and proteins, and altering their functions. The shift in this balance can be triggered by the level of antioxidant or pro-oxidant nutrients in the diet (Martinez-Alvarez et al., 2005). In fish, although little studied, the effect of dietary FM replacement by plant proteins on the oxidative status seems to be dependent on the level and type of the plant ingredient. Antioxidant compounds found in certain plant proteins may reduce oxidative damage (Sitjá-Bobadilla et al., 2005; Olsvik et al., 2011;) while pro-oxidant compounds may induce oxidative stress (Enes et al., 2012; Zheng et al., 2012; Deng et al., 2013). Moreover, dietary content in NSP contents (Enes et al., 2012) or the source of non-protein energy (starch and lipids) may also affect fish antioxidant status (Rueda-Jasso et al., 2004; Castro et al., 2012, 2015, 2016). In present study, despite the increased dietary content of NSP levels and the reduction of protein and energy available for retention, no effect on the liver and intestine antioxidant status was observed. Previously, it was observed that 30% of feed restriction did not affect turbot oxidative status (Abele et al., 2007).

The activities of the antioxidant enzymes and lipid peroxidation markers was considerably higher in turbot intestine than in the liver. The liver and intestine are involved in different functions, and confronted to different challenges, and display distinct antioxidant mechanism strategies (Castro et al., 2015, 2016; Coutinho et al., 2016, 2017; Wu et al., 2017). The present results may be associated with a higher capacity of antioxidant mechanisms in the liver than in the intestine or with a higher diet-induced oxidative damage in the intestine. Indeed, the intestine is the first organ to encounter a number of nutritional insults, thus higher stress response trigged by diet components would most likely be observed here.

The present results indicate that the dietary inclusion of DDGS to a 40% FM based diet for turbot juveniles reduced growth performance and impaired overall nitrogen and energy metabolism. Moreover, turbot susceptibility to oxidative stress was higher in the intestine than in liver, although oxidative status was not affected by dietary DDGS levels.

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Chapter 3

Exogenous enzymes supplementation enhances diet digestibility and digestive function and affects the intestinal microbiota of turbot (*Scophthalmus maximus*) juveniles fed distillers' dried grains with solubles (DDGS) based diets

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ABSTRACT

A trial was conducted to evaluate the effect of dietary exogenous enzymes supplementation on apparent digestibility of macronutrients, energy and amino acids, and on digestive enzymes activity and gut microbiota of turbot (*Scophthalmus maximus*) juveniles fed diets rich in plant feedstuffs. For that purpose, a control diet was formulated to contain circa half protein from fishmeal and the other half from plant feedstuffs (dried distillers' grains with solubles, soybean meal, corn gluten, and wheat gluten). Two other diets were formulated similar to the control but including exogenous enzyme complexes: Synergen® (Alltech; product of solid state fermentation of *Aspergillus niger*) or Natugrain®TS (BASF; non-starch polysaccharides degrading enzymes complex). Both exogenous enzyme complexes increased the apparent digestibility coefficient (ADC) of dry matter, while the ADC of protein, lipid, and energy were only increased with Natugrain®TS. Moreover, the ADC of methionine, isoleucine, and aspartic acid were increased with both exoenzymes supplementation, while the ADC of lysine and glycine were increased with Synergen® and the ADC of arginine and threonine were increased with Natugrain®TS. Dietary supplementation with Synergen® or Natugrain®TS did not affect intestinal pH but increased the activity of lipase and protease in the posterior intestine, while amilase activity was increased only with Natugrain®TS. Microbiota was also affected by both exoenzymes complexes, increasing its richness and diversity. Present results indicate that diet supplementation with exogenous enzymes complexes, Synergen® or Natugrain®TS, may be a feasible strategy to enhance the digestibility of plant feedstuff-rich diets for turbot juveniles.

Keywords: digestibility, digestive enzymes, exogenous enzymes, flatfish, microbiota, plant feedstuffs.

1. Introduction

One of the major challenges for carnivorous fish aquafeeds production is the reduction of dependency on fishery-derived products, namely of fishmeal (FM) and fish oil. Aquafeeds for turbot (*Scophthalmus maximus*) usually include high amount of FM as main protein source, complemented with some plant protein concentrates (Fournier et al., 2004; Bonaldo et al., 2011). More recently, Bonaldo et al. (2015) observed that FM may be replaced my traditional plant feedstuffs (wheat gluten, soybean meal and soy protein concentrate), from 50 to 35% of the diet, with no significant effect on specific growth rate and feed efficiency. However, the use of these plant feedstuffs imposes some concerns due to the "food-feed competition", rising prices, and carbon footprint involved in their production and importation. Thus, there is an increasing need to look for alternatives, particularly underutilized commodities, such as by-products obtained from food, fermentation and pharmaceutical industries. This would have special interested, particularly for the sustainability of European aquaculture, dependent of the imported traditional plant feedstuffs, as soybean meals (Matos et al., 2016). Within these alternative plant feedstuffs, we can consider distillers' dried grains with solubles (DDGS), which are the by-product of cereal distillation for ethanol production. Except for the starch fraction, which is consumed during fermentation, DGGS's nutrient content is almost 3 times more concentrated than the original grain, thus containing higher protein, lipid and fiber levels (Rosentrater and Muthukumarappan, 2006; Liu, 2011). The protein content in DDGS ranges from 25 to 45%, depending on the grain source, with little presence of antinutritional factors commonly found in most plant protein sources. Studies on DDGS incorporation in aquafeeds studies were mainly done in omnivorous fish species, such as channel catfish (*Ictalurus punctatus*; Webster et al., 1991; Lim et al., 2009; Li et al., 2011a), Nile tilapia (*Oreochromis niloticus*) and hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) (Wu et al., 1997; Li et al., 2011b; Schaeffer et al., 2012; Welker et al., 2014a). So far, studies performed on the potential use of DDGS in carnivorous species are limited to a few studies with rainbow trout (*Oncorhynchus mykiss*) (Overland et al., 2013; Welker et al., 2014b), olive flounder (*Paralichthys olivaceus*) (Rahman et al., 2015), European seabass (*Dicentrarchus labrax*) and meagre (*Argyrosomus regius)* (Magalhães et al., 2016). For another high carnivorous flat fish, as olive flounder it was observed that up to 21% of rice based DDGS (with 31% of protein) may be used to replace FM in the diet (Bae et al., 2015).

Insoluble fiber is more resistant to intestinal fermentation than that of more soluble non-starch polysaccharides (NSP), being one of the main factors limiting the

incorporation of low-protein content plant feedstuffs, as DDGS, in carnivorous fish diets. Processing treatments, such as dry and wet fractionation and extrusion, have little efficiency in reducing NSP content of plant feedstuffs and to improve its nutritional value (U.S. Grains Council, 2012; Welker et al., 2014b). A promising strategy to increase the nutritional value of high content NSP plant feedstuffs is the incorporation of exogenous enzymes to break down NSP.

The use of exogenous enzymes as feed additives has been extensively studied for poultry and pig feed industries, and their dietary incorporation is nowadays a common practice to improve digestibility of nutrients, reduce anti-nutritional effects of non-starch polysaccharides (NSP) and phytic acid (Adeola and Cowieson, 2011; Bedford and Cowieson, 2012). However, for aquaculture species, research on exogenous enzyme supplementation has been focused on phytase (Kumar et al., 2012), and only recently interest on dietary carbohydrases supplementation has emerged (revised by Castillo and Gatlin, 2015).

Due to the relative high levels of indigestible NSP in DDGS and soybean, that limit organic matter, energy and protein digestibility, supplementation of diets including these feedstuffs with exogenous enzymes may increase their nutritional value. Indeed, it was recently shown in rainbow trout that dietary exoenzymes incorporation improved breakdown of soybean NSP (Dalsgaard et al., 2012, 2016) and supplementation of a DDGS based-diet with phytase improved phosphorus and essential amino acids digestibility (Cheng and Hardy, 2004).

Therefore, the aim of the present study was to evaluate the effect of exogenous enzymes supplementation of a plant feedstuffs-rich diet on nutrient digestibility, digestive enzymes activities, and gut microbiota of turbot.

2. Materials and Methods

This study was directed by accredited scientists (following FELASA category C recommendations) and conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes.

2.1 Experimental diets

A control diet meeting the nutritional requirements of turbot (*Scophthalmus maximus*) juveniles was formulated to include circa half of protein from fishmeal (FM) and the other half from plant feedstuffs (corn-DDGS, soybean meal, corn gluten, and

Table 1. Formulation and proximate composition (% dry matter) of the experimental diets.

Diet	Control	SYN	NAT
Ingredients			
Fish meal[a]	32.2	32.2	32.2
DDGS[b]	25	25	25
Wheat gluten[c]	5	5	5
Corn gluten[d]	15	15	15
Soybean meal[e]	10	10	10
Wheat meal[f]	0.7	0.65	0.65
Fish oil	8.6	8.6	8.6
Vitamin premix[g]	1	1	1
Choline chloride (50%)	0.5	0.5	0.5
Mineral premix[h]	1	1	1
Binder[i]	1	1	1
SYN[j]	—	0.04	—
NAT[k]	—	—	0.04
Proximate composition			
Dry matter (%)	86.3	87.6	85.9
Crude protein	53.7	54.1	54.6
Crude lipid	14.8	15.2	15.4
Ash	9.4	9.3	9.3
NFE[l]	22.1	21.4	20.7
Gross energy (kJ g^{-1})	22.9	23.5	22.2
Essential amino acids			
Lysine	3.28	3.15	3.18
Arginine	3.65	3.74	3.75
Histidine	1.89	1.91	1.89
Isoleucine	2.55	2.71	2.72
Leucine	5.86	5.86	5.23
Valine	2.91	2.91	3.27
Methionine	1.36	1.40	1.41
Phenylalanine	2.84	2.84	2.93
Threonine	2.38	2.38	2.28
Non-essential amino acids			
Tyrosine	2.27	2.27	2.29
Aspartic Acid	3.86	4.08	4.37
Glutamic Acid	8.74	9.17	9.21
Serine	2.27	2.33	2.34
Glycine	2.48	2.26	2.59
Alanine	3.52	3.51	3.59
Proline	3.31	3.31	3.31

[a]Pesquera Centinela, Steam Dried LT, Chile (CP: 74.2%; CL 10.1%). Sorgal, S.A. Ovar, Portugal.
[b]DDGS (CP: 32.8%; CL:9.0%; Starch: 0.5%; Acid detergent fiber: 14.0; Neutral detergent fiber: 40.8) Pannonia Gold®.
[c]Wheat gluten (CP: 84.3%; CL: 3.9%), Sorgal, S.A. Ovar, Portugal.
[d]Corn gluten (CP: 68.3%; CL: 2.9%), Sorgal, S.A. Ovar, Portugal.
[e]Soybean meal (CP: 53.7%; CL:2.1%), Sorgal, S.A. Ovar, Portugal.
[f]Wheat meal (CP: 14.6%; CL:2.2%), Sorgal, S.A. Ovar, Portugal.
[g]Vitamins (mg kg^{-1} diet): retinol, 18000 (IU kg^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.
[h]Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.4 (g kg^{-1} diet).
[i]Aquacube. Agil, UK.
[j]Synergen, Alltech, Kentucky, USA.
[k]Natrugain®TS, BASF, Germany.
[l]Nitrogen free extract = 100 − (crude protein + crude lipid + ash)

wheat gluten). Two other diets were formulated similar to the control diet but including two commercial exogenous enzymes complexes: Synergen™, Alltech (SYN) or Natugrain®TS, BASF (NAT). SYN is a natural solid-state fermentation complex of *Aspergillus niger* that contains residual enzyme activity (www.altech.com). NAT is a feed enzyme complex of highly purified non-starch polysaccharides enzymes, namely endo-1,4-beta-xylanase (5600 TXU/g) and endo-1,4-beta-glucanase (2500 TGU/g). Both exoenzymes were add before pelleting, as they are recommended for pelleted feed up to 85°C (BASF recommendations). Chromic oxide was incorporated to the diets as inert digestibility marker. All ingredients were finely ground, thoroughly mixed, and pelleted using a laboratory pellet mill (CPM: California Pellet Mill, Crowfordsville, IN, USA) through a 2 mm dye. Diets were dried in an oven for 24 h at 40 °C and then stored at -20 ºC until use. The formulation and proximate composition of the diets are presented in Table 1.

2.2 *Digestibility trial*

The digestibility trial was performed at the Marine Zoological Station, University of Porto, Portugal, with turbot juveniles provided by a commercial aquaculture. The experimental system consisted of a thermoregulated recirculating water system equipped with nine fiberglass tanks (60 l water capacity) with feces settling column connected to the outlet of each tank. Both tanks and settling columns were designed according Cho et al. (1982). During the trial, water flow was established at about 4.5 l min^{-1} per tank, water temperature was 18±1 °C and water salinity 35‰, dissolved oxygen was kept near saturation and nitrogenous compounds maintained below 0.02 mg^{-1}. Photoperiod was adjusted to 12 h light as 12 h dark.

After a quarantine period of one month, fish were transferred to the experimental system and adapted to the experimental conditions for 15 days. At the beginning of the trial, nine homogenous groups of 15 fish with an average weight of 72 g were established. The experimental diets were randomly assigned to triplicate groups and fish were daily fed by hand, twice a day, at 9:00h and 16:00h. After 10 days of adaptation to the experimental diets, feces were collected once a day during 22 consecutive days. Feces accumulated in each settling column were collected before the morning meal, immediately centrifuged at 3 000 x g for 15 minutes, pooled for each tank and stored at -20 °C until analysis. One hour after the afternoon meal, tanks, pipes, and settling columns were thoroughly cleaned to remove feces and uneaten feed. Apparent digestibility coefficients (ADC) of dry matter, lipids, protein, amino acids, and energy of the experimental diets were calculated as follows:

$$\mathrm{ADC_{diet}} = \left(1 - \left(\frac{\text{dietary } \mathrm{Cr_2O_3} \text{ level} \times \text{feces nutrient or energy level}}{(\text{feces } \mathrm{Cr_2O_3} \text{ level} \times \text{dietary nutrient or energy level})}\right)\right) \times 100$$

2.3 Intestine sampling

At the end of the digestibility trial, on the sampling day, fish were continuously fed and thereafter three fish per tank were randomly sampled for digestive enzymes activity measurement. Only fish with digesta along the intestine were sampled to ensure intestinal exposure to the diets (Krogdahl and Bakke, 2005). Fish were euthanized with a sharp blow to the head and immediately eviscerated on an ice-cooled tray. Intestine was excised, adherent adipose and connective tissues were carefully removed, and the intestine divided in three portions: anterior, mid, and posterior. The posterior intestine was distinguished from de mid intestine by increased diameter, darker mucosa, and annular rings. The anterior and mid portions of the intestine were obtained by division of the remaining intestine in two identical sections. The anterior intestine is the portion directly after the stomach and included the pyloric caeca. The individual pH of each intestine section was determined in situ using a pH meter (pH Eutech Instrument) and the intestine portions were then immediately frozen in liquid nitrogen and stored at -80°C until use.

Another two fish per tank were euthanized for intestinal microbiota analysis characterization. The entire intestine was aseptically removed from the abdominal cavity and scrapped with tweezers for collection of digesta content. Samples were placed on sterile tubes, immediately frozen in liquid nitrogen, and stored at -80 °C until used.

2.4 Chemical analysis

Analysis of ingredients, diets, and feces were conducted as follows: dry matter, by drying the samples at 105 °C until constant weight; protein content (N x 6.25) by the Kjeldahl method following acid digestion, using Kjeltec digestion and distillation units (Tecator Systems, Höganäs, Sweden; models 1015 and 1026, respectively); lipid content by extraction with petroleum ether using a Soxtec system (Tecator Systems, Höganäs, Sweden; extraction unit model 1043 and service unit model 1046); ash by incineration in a muffle furnace at 450 °C for 16 h; gross energy by direct combustion of samples in an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261); chromic oxide by acid digestion according to Furukawa and Tsukahara (1966). Amino acids were analysed by high pressure liquid chromatography (HPLC) in a Pico-Tag Amino Acid Analysis System (Waters auto sample model 717 plus; Waters binary pump model 1525; Waters dual absorbance detector model 2487; Waters, USA),

using norleucine as internal standard, according to the procedures described by Banuelos-Vargas et al. (2014).

2.5 *Digestive enzyme activities*

Each intestine section tract was homogenized in ice-cold buffer, at pH 7.8 (the averaged intestine´s pH of experimental fish according to Table 3) and centrifuged at 30 000g for 30 min at 4 °C. The resultant supernatants were collected and aliquots were stored at -80 °C until digestive enzyme analysis. α-Amylase (EC 3.2.1.1) activity was determined with a commercial kit (ref. 41201, Spinreact, Girona, Spain) with modifications; the rate of product formation (2-chloro-4-nitrophenol) was quantified at 405 nm. Lipase (EC 3.1.1.3) activity was determined using a commercial kit (ref. 1001275, Spinreact, Girona, Spain) with modifications; 1-2-O-dilauryl- rac-glycero-3-glutaric acid-60-methylresorufin-ester was used as substrate, and the formation rate of methylresorufin was followed at 580 nm. Total protease activity was measured by the casein-hydrolysis method. A reaction mixture containing casein at 1% (w/v), buffer (0.1 M Tris HCl at pH 7.8) and supernatant from the homogenates was incubated for 1 h at 37 °C, stopped by adding trichloroacetic acid solution (8%; w/v); kept for 1 h at 2 °C, centrifuged at 1800 g for 10 min and the supernatant absorbance was measured at 280 nm against blanks. A control blank for each sample was assayed adding the supernatant from the homogenates after the incubation time. Tyrosine solution was used as standard.

Unit (U) of enzyme activity was defined as µmol of product generated per minute under the measurement conditions described above and expressed per mg soluble protein (specific activity). Protein concentration was determined using Bradford's method (1976), with bovine serum albumin solution as standard.

2.6 DNA extraction from fecal samples

DNA extraction was performed by homogenizing 300mg of digesta (from a pool of 2 fish per tank to reduce variation) in 500 µl STE buffer (0.1 M NaCl, 10 mM Tris, 1mmM EDTA, pH 8) containing 0.4 g of glass beads (Sigma-Aldrich, G8772). Homogenization was done twice for 30 s, with an interval of at least 30 s on ice, on a BeadBug bead-beater (Benchmark Scientific, Edison, NJ, USA) at a speed of 2 500 g. Following 15 min incubation at 75 °C, with gentle agitation every 5 min, glass beads were removed by centrifugation and DNA extraction was continued according to the method described by Pitcher et al. (1989) with some modifications. Briefly, samples were sequentially incubated for 30 min at 37 °C in the presence of 50 mg/ml of lysozyme followed by the addition of 10mg/ml RNAse. A third 30 min incubation was done at 55 °C with 20 mg/ml

Proteinase K and 10% SDS. After 10 min on ice in the presence of 500 µl of GES (Pitcher et al, 1989) and 250 µl of ammonium acetate (7.5 M), a phenol-chloroform extraction was performed by adding 500 µl phenol-chloroform-isoamyl alcohol (25:24:1). The aqueous phase was re-extracted with 500 µl of chloroform-isoamyl alcohol (24:1) and the DNA of the subsequent aqueous phase was precipitated with 0.6 volumes of isopropanol. After 10 min centrifugation at 13 000 g the DNA pellet was washed with ice-cold 80 % ethanol and dried at room temperature. DNA was ressuspended in 100 µl ultrapure water.

2.7 Polymorphism analyses of 16S rRNA genes by denaturing gradient gel electrophoresis (DGGE)

Bacterial 16S rDNA gene fragments were amplified by a touchdown PCR on a T100TM Thermal Cycler (Bio-Rad), using primers 16S-358F (which has a GC clamp at the 5´end) and 16S-517R (Muyzer et al, 1993), yielding a 233bp DNA fragment. PCR mixtures (50 µl) containing 24.75 µl of water (Sigma), 10 µl of GoTaq Buffer 5X (PROMEGA), 5 µl of each dNTPs (2 mM, PROMEGA), 2.5 µl of each primer (10 µM Forward and Reverse), 0.25 µl of GoTaq polymerase (PROMEGA), and 5 µl of DNA template were subjected to a touchdown PCR. A 94 °C incubation for 5 min was followed by 10 cycles of 64 °C for 1 min, 65ºC for 1 min and 72 °C for 3 min. The annealing temperature was decreased 1 °C per cycle, until reach 55 °C. Finally, 20 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min. Final extension was at 72 °C, 10 min. PCR products were resolved by electrophoresis on 1 % (w/v) agarose gels containing Gel Red (Biotium) to check for product size. DNA concentration was quantified with the µDropTM Plate (Thermo Fisher Scientific Inc.) and 300ng of each PCR product was loaded on an 8% polyacrylamide gel composed by a denaturing gradient of 40 to 80% 7M urea/40% formamide. Electrophoresis occurred on a DCode™ universal mutation detection system (Bio-Rad), during 16h at 60°C, 65V in 1×TAE buffer. Gels were stained for 1h with SYBR-Gold Nucleic Acid Gel Stain, and imaged on a Gel Doc EZ System (Bio-Rad) with the Image Lab software v4.0.1 (Bio-Rad). Selected bands were excised from the gel and eluted in 20 µl ultrapure water prior to DNA re-amplification using the same oligonucleotide primers as above, but without the GC clamp. Amplicons were sequenced to identify microbiota OTUs (Operational Taxonomic Units). Phylogenetic analysis, to identify the closest known species, was done by comparison with sequences in the GenBank non-redundant nucleotide database using BLAST (http://www.ncbi.nlm.nih.gov). Only sequences higher than 100 bp reads and 80–100% query coverage were considered a valid identification.

2.8 Statistical analysis

Data were checked for normality and homoscedasticity and if needed normalized (log normalization or arcsine square root transformation). DGGE banding patterns were transformed into presence/absence matrices and band intensities measured using Quantity One 1-D Analysis Software v4.6.9 (Bio-Rad Laboratories, Lda. Amadora, Portugal). Relative similarities between dietary treatments and replicates were calculated using Primer software v7.0.5 (PRIMER-E Ltd, Ivybridge, UK). Similarity percentages (SIMPER) were used to represent the relative similarities between treatments. Species richness was assessed using Margalef's measure of richness, and species diversity was assessed by the Shannon–Weaver index. Clustering of DGGE patterns was achieved by construction of dendrograms using the Unweighted Pair Groups Method with Arithmetic Averages (UPGMA). Multivariate analysis was done using the metric multidimensional scaling (MDS) with ordinations based on Bray-Curtis similarities utilizing relative band abundances. MDS data representation was considered reliable based on the Kruskal stress values (< 0.2) calculated (Clarke and Warwick, 2001). The effect of the dietary treatments on the different parameters was analyzed by one-way ANOVA. Significant differences among means ($P<0.05$) were determined by the Tukey's multiple range test. All statistical analyses were carried out using the SPSS version 22.0 software package.

3. Results

The apparent digestibility coefficients (ADC) of nutrients and energy of the experimental diets are presented in Table 2. Both exogenous enzyme complexes increased dry matter digestibility, which was higher for the NAT than the SYN diet. Although there was a trend for an increase of the ADC of protein, lipids, and energy with both exogenous enzyme complexes, only with NAT the ADC were significantly higher than with the control diet. Comparatively to the control, the ADC of methionine, isoleucine, and aspartic acid were higher in SYN and NAT diets, while lysine and glycine were higher only in the SYN diet, and arginine and threonine were higher only in the NAT diet.

pH and specific activity of digestive enzymes in the anterior, mid and posterior intestine sections are presented in Table 3. Dietary supplementation with SYN or NAT did not affect intestinal pH, which increased along the intestine, irrespectively of the dietary treatment, from an average of 7.5 in the anterior intestine to 8.0 in the posterior

Table 2. Apparent digestibility coefficients (%) of nutrients and energy of the experimental diets.

Diets	Control	SYN	NAT	SEM
Dry matter	61.1^{a}	64.7^{b}	69.8^{c}	1.5
Protein	86.4^{a}	87.5^{ab}	89.8^{b}	0.6
Lipids	65.7^{a}	69.7^{ab}	73.8^{b}	1.4
Energy	78.5^{a}	79.4^{a}	84.4^{b}	1.0
Essential amino acids				
Lysine	81.1^{a}	90.4^{b}	85.3^{ab}	4.7
Arginine	80.7^{a}	86.8^{ab}	87.1^{b}	3.7
Histidine	81.2	84.7	84.5	2.6
Isoleucine	79.8^{a}	85.1^{b}	85.1^{b}	3.2
Leucine	88.0	88.2	90.7	2.1
Valine	83.7	85.0	84.8	2.4
Methionine	81.5^{a}	90.2^{b}	90.5^{b}	4.9
Phenylalanine	81.3	86.1	86.7	3.3
Threonine	80.7^{a}	83.4^{ab}	90.8^{b}	5.7
Non-essential amino acids				
Tyrosine	82.1	85.4	80.6	3.5
Aspartic Acid	81.3^{a}	89.3^{b}	90.2^{b}	4.8
Glutamic Acid	88.1	91.2	91.4	2.0
Serine	80.0	83.3	81.5	2.8
Glycine	83.3^{a}	88.0^{b}	84.4^{ab}	2.6
Alanine	83.3	83.1	82.8	3.5
Proline	81.9	82.2	80.9	1.9

Values presented as means (n = 3) and pooled standard error of the mean (SEM).
Means in the same row with different letters are significantly different ($P < 0.05$).

Table 3. pH and specific activity (mU mg protein^{-1}) of amylase, lipase and proteases in the anterior, mid and posterior intestine of turbot juveniles fed experimental diets.

Diets	Control	SYN	NAT	SEM
pH				
Anterior	^{A}7.4	^{A}7.6	^{A}7.6	0.56
Mid	^{B}7.9	^{AB}7.8	^{AB}7.8	0.54
Posterior	^{B}8.0	^{B}8.1	^{B}7.9	0.30
AVERAGE	7.8	7.8	7.8	0.07
Amylase				
Anterior	^{AB}6.8^{b}	^{A}5.7^{a}	^{A}7.9^{c}	0.2
Mid	^{B}7.5^{a}	^{B}8.2^{ab}	^{B}9.7^{b}	0.4
Posterior	^{A}5.2^{a}	^{B}7.8^{b}	^{A}6.8^{ab}	0.4
SUM	19.5^{a}	21.8^{ab}	24.4^{b}	0.7
Lipase				
Anterior	^{AB}3.8	^{A}3.7	^{A}3.6	0.2
Mid	^{B}4.5	^{B}5.8	^{B}5.2	0.3
Posterior	^{A}3.1^{a}	^{B}5.3^{b}	^{AB}4.8^{b}	0.2
SUM	11.5^{a}	15.3^{b}	13.6^{b}	0.5
Protease				
Anterior	^{A}166.5	^{A}139.5	^{A}166.5	10.4
Mid	^{B}294.2	^{B}270.7	^{B}291.2	11.8
Posterior	^{B}256.5^{a}	^{C}393.6^{b}	^{B}360.9^{b}	18.3
SUM	717.2	803.8	809.4	19.2

Values presented as means (n = 9) and pooled standard error of the mean (SEM). In the same column, different uppercase letters indicate significant differences between intestinal sections; in the same row, different lowercase letters indicate significant differences between dietary treatments ($P<0.05$).

intestine. There was no clear trend of amylase activity along the intestinal sections, but sum of amylase activity was higher in fish fed the NAT diet than the control diet. Lipase and protease activities in the anterior and mid intestine were little affected by diet composition, but in the posterior intestine both enzymes activities were higher in fish fed the experimental diets than in the control. Sum of lipase activity was higher in fish fed the experimental diets than in the control, but there were no differences between groups in protease activity

The microbial community profiling of the intestinal contents recovered from turbot fed the experimental diets was studied by polymorphism analyses of the variable V3 region of the 16S rRNA gene using DGGE. Similar banding patterns were found in 2 out of 3 replicates for each feeding condition, with one replicate constantly failing to cluster with the other two in the Bray–Curtis dendrogram (Figure 1). The figure further shows

Table 4. Ecological parameters obtained from PCR-DGGE fingerprints of the intestinal allochthonous microbiota of turbot juveniles fed the experimental diets.

Diets	Control	SYN	NAT	SEM
OTUs[a]	7.33	12.0	12.7	1.2
Richness[b]	0.42	0.68	0.73	0.1
Diversity[c]	1.93	2.39	2.50	0.1
SIMPER Similarity (%)[d]	58.8	43.2	62.9	6.3

Values presented as means (n = 3, pooled from 6 fish) and pooled standard error of the mean (SEM).
[a]OTUs: Average number of operational taxonomic units.
[b]Margalef species richness: d=(S-1)/ log(N)
[c]Shannons diversity index: H'= -∑(pi × (Lnpi))
[d]SIMPER: similarity percentage within group replicates.

that bacterial communities obtained from fish fed the SYN supplemented diet seem to be more closely related (percentages of similarity higher than 80% between 2 out of 3 samples) than those recovered from fish fed the control or NAT supplemented diet (percentages of similarity around 70% between 2 out of 3 samples). The presence of a replicate that did not cluster with the other 2 in each experimental condition led to high variability on the number of OTUs, microbial richness, diversity and similarity indices that precluded statistically significant differences between experimental diets (Table 4). Nevertheless, a tendency to an increase in the OTUs, microbial richness and diversity was observed with the SYN or NAT diets. Also, the multidimensional scaling (MDS) plot (Figure 2) revealed that, although there is an overlap of 50% similarity between the different experimental conditions, SYN samples are more similar between each other (70% of similarity).

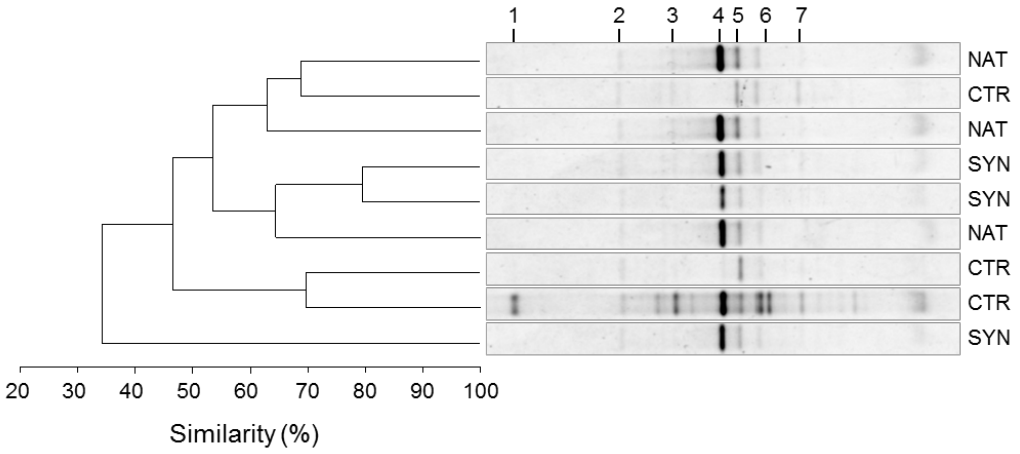

Figure 1. Dendrograms and PCR-DGGE fingerprints of the allochthonous intestinal microbiota of turbot juveniles fed the experimental diets. Numbers on top the Figure (1 to 7) indicate bands excised for sequence analysis (CRT; NAT and SYN: control, NAT and SYN diets, respectively).

Sequence analysis from the DGGE bands (Table 5) showed that the detectable dominant bacteria present in turbot intestine were most closely related to uncultured bacteria or bacteria belonging to the *Lactobacillus*, *Vibrio*, *Pseudomonas* or *Bacillus* genera. The band corresponding to *Vibrio* sp. was present in all replicates of the exoenzymes supplemented diets, but only in one of the control diet replicates.

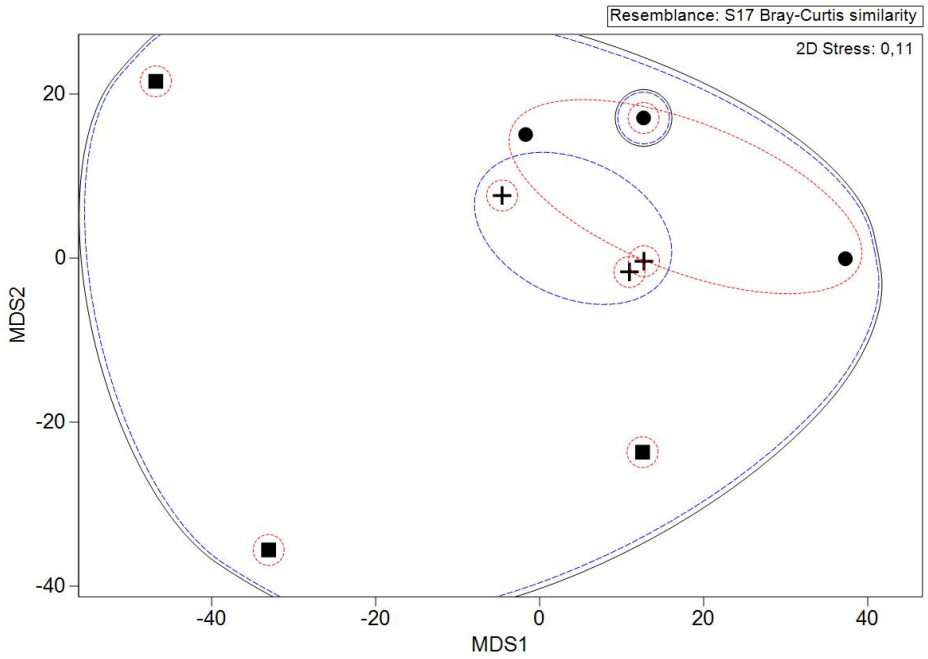

Figure 2. Multidimensional scaling (MDS) plot of DGGE bands presence and abundances of turbot juveniles fed the same experimental diets depicted in Figure 1: (+) Control; (●) SYN; (■) NAT diets. Black continuous line, blue dotted and red dotted lines represent 45, 50 and 70% similarity between samples, respectively.

Table 5. Closest relatives (BLAST) to the sequenced PCR-DGGE gel bands of the intestinal allochthonous communities of turbot juveniles fed the experimental diets.

Band	Nearest neighbor	Similarity to nearest neighbor	Accession number of nearest neighbor
1	*Lactobacillus aviarius* subsp. araffinosus strain LMG 23560 16S ribosomal RNA gene. partial sequence	98	NR_104979.1
2	Uncultured bacterium isolate DGGE gel band G13 16S ribosomal RNA gene. partial sequence	98	HM216398.1
3	*Pseudomonas sp.* 2085 16S ribosomal RNA gene. partial sequence	83	KC236522.1
4	*Vibrio sp.* VibP-Oc-139 16S ribosomal RNA gene. partial sequence	96	KF577005.1
5	Uncultured Bacilli bacterium clone MS046A1_G04 16S ribosomal RNA gene. partial sequence	87	EF701997.1
6	Bacillus amyloliquefaciens strain PPL-S7 16S ribosomal RNA gene. partial sequence	89	KM226906.1
7	Uncultured bacterium clone TCB1 16S ribosomal RNA gene. partial sequence	95	AF392785.1

4. Discussion

Success of FM replacement by plant feedstuffs seems to be more limited in turbot than in other marine carnivorous fish species (Davies et al., 2009; Bonaldo et al., 2011; Nagel et al., 2012). Turbot seems to digest poorly diets including high levels of plant feedstuffs when compared to FM-based diets (von Danwitz et al., 2016; Regost et al., 1999; Nagel et al., 2012). Turbot´s shorter gastro-intestinal tract and lower rearing temperature were pointed out as limiting digestive capacity to utilize plant feedstuffs (Davis et al., 2009). Thus, development of strategies that improve digestive utilization of plant feedstuffs are critical for turbot aquafeed industry.

The low ADC values of dry matter and energy of the control diet may in part be related to the high level of nitrogen free extract, which is mainly composed by NSP and thus it is not digestible by carnivorous fish as they lack the enzymes needed digest it (Krogdahl et al., 2005). Besides being an indigestible diet fraction, NSP form a gum-like mass that increases digesta viscosity, impairing the action of digestive enzymes, limiting their access to subtracts and so reducing overall nutrients digestibility (Castillo and Gatlin, 2015; Sinha et al., 2011). Moreover, NSP may bind bile acids, obstruct microvilli,

and impair the movement of digesta along the digestive tract (Castillo and Gatlin, 2015). This may contribute to explain the reduced ADC observed in this study.

To overcome these constraints, supplementation of diets with exogenous enzymes that digest NSP may be a strategy to improve nutrient digestibility of plant-based diets (Bedford, 2000; Castillo and Gatlin, 2015). However, the efficacy of exoenzymes depends on the amount and characteristics of dietary feedstuffs, type and dietary concentration of enzymes, fish species and size, and water temperature (Castillo and Gatlin, 2015). Up to now, the use of phytase to improve phosphorus availability in fish diets has been successfully demonstrated (Kumar et al., 2012), inclusively in turbot (von Danwitz et al., 2016), but the potential of using other exoenzymes is much less studied (Castillo and Gatlin, 2015).

The two commercial exoenzymes complexes tested in the present study were chosen based on their characteristics. While NAT is a complex of highly purified non-starch polysaccharidases (NSPases; xylanase and glucanase) and therefore was mostly directed to the hydrolysis of DDGS and soybean meal NSP of the experimental diets, SYN is a by-product of solid-state fermentation, containing phytase, proteases, lipases, and carbohydrases that are expected to work synergistically to improve nutrient availability.

In this study, dietary SYN supplementation lead to an increase of the ADC of dry matter, but not of protein, lipids or energy. Although the ADC of protein was not affected by dietary supplementation with SYN, the ADC of some AA was improved (lysine, isoleucine, methionine, aspartic acid and glycine) suggesting a positive effect of SYN's proteases. Dietary NAT supplementation was more efficient in improving overall diet digestibility, as it increased the ADC of dry matter, protein, lipids and energy of the experimental diets, as well as the ADC of methionine, arginine, isoleucine, threonine and aspartic acid. This suggests that NAT supplementation promoted the hydrolysis of NSP, which explains the increase of dry matter and energy digestibility. By hydrolyzing NSP, digesta viscosity might also have been reduced and bile acids might become more available, facilitating the access of endogenous digestive enzymes to nutrients, this contributing the overall increase of nutrients digestibility (Castillo and Gatlin, 2015; Dalsgaard et al., 2012; Gatlin et al., 2007).

In a previous study with seabass, both exoenzymes complexes were tested at higher dietary inclusion levels (1 or 2%) in diets including 20% FM protein and 80% of plant protein (wheat gluten, corn gluten, soybean meal, pea protein concentrate, wheat) (Díaz-Rosales et al., 2014). These enzyme inclusion levels were considerably higher

than the supplementation level recommended by the supplier, which is more similar to the values used in the present study. Nevertheless, in that study NAT improved the ADC of dry matter, protein and lipids at both levels tested, while SYN failed to improve the ADC of lipids and only improved the ADC of protein at the highest level tested. On the contrary, in white seabream fed diets including just 3.5 or 10% FM protein and the rest provided by plant feedstuffs (soybean meal, corn gluten, pea protein concentrate, wheat), dietary supplementation with NAT at the same concentration used in the present trial did not improve ADC of protein, energy or phosphorus (Magalhães et al. 2016). These results highlight the need to carefully evaluate the benefits of different exoenzyme complexes according to species, diet formulation and enzyme supplementation level. Moreover, besides species-specific differences in digestive machinery capability, differences in rearing temperature may also contribute to explain the differences in exoenzymes efficacy between turbot and seabass. Indeed, most commercial enzymes were developed for homoeothermic animals and are more active at high temperatures. As turbot is reared at lower water temperatures than seabass (18 and 24°C, respectively), this may contribute to e effectiveness of exogenous enzymes may be lower for this species. This highlight the pivotal importance of understanding the potential and limitations of exoenzymes, as if inappropriately applied it will lead to waste of resources (Adeola and Cowieson, 2011).

The increase of the ADC of lysine with SYN or of the ADC of methionine, arginine and threonine with NAT, may be of particular interest for diet formulation, as these amino acids are among the first limiting essential amino acids in plant feedstuffs (Oliva-Teles et al., 2015). Thus, increasing their bioavailability may allow more flexible feed formulation for turbot, which has high dietary essential amino acid requirements (Peres and Oliva-Teles, 2008).

Even though knowledge on digestive processes regulation in fish is fragmentary, it is generally accepted that diet composition influences digestive enzyme secretion (revised by Krogdahl et al., 2015) and that dietary NSP may limit overall digestive enzyme activity (Sinha et al., 2011). As aforementioned, hydrolysis of NSP reduces digesta viscosity and reduces the binding of NSP with bile salts, thus increasing bile salts availability in the intestinal gut, which is essential for the activation of lipase and emulsification of lipids (Castillo and Gatlin, 2015).

In the present study, lipase activity, along the intestinal tract, and protease activity, in the posterior intestine, increased with both exoenzymes supplementation, while amylase activity increased with NAT supplementation in the anterior and mid

intestine and with SYN supplementation and in the posterior intestine. Overall, sum of enzymes activities was also higher in the exoenzymes supplemented diets than in the control, though this increase was only significant for amylase and lipase with NAT and for lipase with SYN. This seems to indicate that reduction of digesta viscosity and increased bile salts availability induced increased enzyme secretion and action along the intestinal tract. Also, as NAT is exclusively composed of NSPases, the increased lipase and protease activities in the intestinal tract of fish fed the diet with this exoenzyme complex is also likely due to an increased availability of nutrients by the digestion of NSP.

Contrary to the present results, in sea bass dietary SYN and NAT supplementation did not affect digestive enzymes activities (Diaz-Rosales et al. 2014) while in white seabream only amylase activity improved with dietary NAT supplementation (Magalhães et al. 2016). Also, in hybrid tilapia dietary supplementation with a NSP enzyme complexes increased amylase but not protease and lipases activities (Li et al., 2009) and in tilapia a β-glucanase and xylanase exoenzyme complex increased amylase and protease activities (Lin et al., 2007). Further, in rohu (*Labeo rohita*) and grass carp (*Ctenopharyngodon idellus*), dietary cellulase supplementation increased proteases, amylase, and lipase activities (Xavier et al., 2012; Zhou et al., 2013) and in Jian carp (*Cyprinus carpio* var. Jian) dietary xylanase supplementation increased trypsin, chymotrypsin, amylase, and lipase activities of (Jiang et al., 2014).

A clear trend of digestive enzyme activity along the intestine was not observed in the present study, except for protease activity, which increased from the anterior to the posterior intestine. In another study also in turbot higher enzyme activities were observed in the mid or posterior intestine than in the anterior intestine (Izquierdo and Henderson, 1998). Studies in other species show contradictory results regarding digestive enzymes activity along the digestive tract. For instance, in seabass and meagre higher enzyme activities were also observed in the mid or posterior intestine than in the anterior intestine (Magalhães et al., 2017, 2015; Pérez-Jiménez et al., 2009), while higher digestive action in the anterior than in the posterior section of the intestine were observed in white seabream, meagre, rainbow trout or Atlantic salmon (Krogdahl et al., 2015; Castro et al., 2013; Gai et al., 2012). Thus, there seems that no clear trend exists in fish regarding enzyme activity along the intestinal tract. According to Izquierdo and Henderson (1998), the increased digestive enzyme activity in the posterior intestine section of turbot may indicate a higher role of the posterior intestine in the digestion process due to turbot´s short intestine length and for presenting only two rudimentary caeca. The lack of a clear trend in enzyme activity observed in the present study may also be somehow artificial

and be related to a possible drag of digestive enzymes along the intestine induced by the viscosity of NSP, as suggested by Magalhães et al. (2015).

pH values along turbot´s intestine are within the range of values reported for the intestinal tract of other fish species (revised by Krogdahl et al., 2015), and although intestine pH got more basic along the intestinal tract, dietary supplementation with exoenzymes did not affect it. The effect of exogenous enzyme on intestinal pH was not previously studied in fish, thus it is not possible to compare our results with studies in other fish. However, an acidification of digesta was observed in terrestrial animals, which was mainly attributed to increased production of short-chain fatty acids (Yi et al., 2013; Högberg and Lindberg, 2004; Agyekum et al., 2016).

In accordance with a previous work, defining turbot gut microbiota taxonomy (Xing et al, 2013), in this study the dominant cultivable bacterial phyla found in turbot intestine were Proteobacteria (*Pseudomonas* sp. and *Vibrio* sp.) and Firmicutes (*Lactobacillus* sp. and *Bacillus* sp.), irrespectively of the dietary treatment. While the last two genera (*Lactobacillus* and *Bacillus*) are often associated with beneficial bacterial species, commonly used as probiotics (Akhter et al, 2015), the first two (*Pseudomonas* and *Vibrio*) are mostly composed by pathogenic species, affecting humans and animals, including fish (Gauthier, 2015). *Pseudomonas* are however frequently detected in the intestine of healthy marine fish (Silva et al., 2011; Sitjà-Bobadilla et al., 2006; Martin-Antonio et al., 2007). *Vibrio* is commonly found in marine environment, and it is inclusively the predominant genus of marine fish microbiota (Olafsen 2001; Eddy and Jones 2002; Sugita et al. 2002; Sugita and Ito 2006; Martin-Antonio et al., 2007; Gatesoupe et al., 2014), including turbot (Cerda-Cuellar and Blanch 2002).

It is generally accepted that fish microbiota changes according to diet composition (Ringo et al., 2006; Silva et al., 2011). However, little is known regarding the effect of dietary exogenous enzymes supplementation in fish microbiota (Adeoye et al., 2016). In terrestrial animal, studies have shown a complex interaction between dietary supplementation of exogenous enzymes, especially NSPases, and intestinal microbiota, generally relating digesta viscosity, dietary soluble NSP, microbial fermentation and microbial diversity (Bedford and Cowieson, 2012). In the present study no clear effect of dietary exoenzymes supplementation in turbot intestinal microbiota was noticed, mainly due to the high variability observed within dietary treatments. Nevertheless, a trend was observed towards microbiota modulation, with an increase of intestine bacterial species diversity and richness. Interestingly, bands 1 and 6, corresponding to *Lactobacillus* and *Bacillus*, respectively, are only present in samples obtained from fish fed the control diet,

and band 4, corresponding to *Vibrio* sp., is present in all replicates of the enzyme supplemented diets but in only one of the control diet replicates. This shift in intestinal microbiota communities may be consequence of residual nutrients available for fermentation as well as competition among microbial assemblages. The reduction of *Lactobacillus* and *Bacillus* in turbot fed the exoenzymes supplemented diets, suggests a reduction of the potential substrates available for bacterial growth, due to the increased diet digestibility and digestive enzymes activity. The shift to an increase of *Vibrio* number in supplemented diets is not clear and may be due to reduction of competition among microbial communities. Similarly, other authors pointed out that the presence of dietary NSP limited opportunistic bacteria (Silva et al., 2011; Gatesoupe et al., 2014). Thus, the reduction of NSP in the digesta through exoenzymes supplementation may have promoted the growth of Vibrio in turbot intestine. A more detailed and extensive study, namely by using high-throughput sequencing analysis of the gut microbiota modulation by SYN and NAT feed supplementation should in future be performed to corroborate this hypothesis.

Overall, present results indicate that dietary supplementation with NAT exogenous enzymes complex enhanced the ADC of dry matter, lipids, protein, energy, and some essential amino acids of turbot juveniles fed diets rich in NSP. Dietary SYN supplementation improved the ADC of dry matter and of some essential amino acids, but not the ADC of lipids, protein and energy. Moreover, dietary exoenzymes supplementation seems to increase digestive enzymes activity and to modulate microbiota, increasing its richness and diversity.

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Chapter 4

Soybean meal replacement by corn distillers dried grains with solubles (DDGS) and exogenous non-starch polysaccharidases supplementation in diets for gilthead seabream (*Sparus aurata*) juveniles

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ABSTRACT

A growth trial was conducted to evaluate the effect of dietary plant-protein replacement by corn distillers dried grains with solubles (DDGS), and of exogenous carbohydrases supplement (Natugrain®TS, BASF) in gilthead seabream (Sparus aurata) juveniles. For that purpose, a control diet was formulated with 35% of fish meal and 20% soybean meal (SBM) as main protein sources. Two other diets were formulated incorporating 15 or 35% of DDGS, replacing 37.5% or 100% of SBM (diets DDGS15 and DDGS35, respectively). Another diet was formulated similar to diet DDGS35 and supplemented with 0.1% of a commercial non-starch polysaccharidases complex, Natugrain®TS, BASF (NAT; diet DDGS35ENZ). Triplicate groups of fish (IBW = 15 ± 1g) were fed the experimental diets for 67 days at 22 °C. At the end of the trial, growth performance, voluntary feed intake, feed efficiency, protein and energy retention were not affected by dietary DDGS incorporation. Dietary NAT supplementation also did not affect growth performance, but increased feed efficiency, nitrogen and energy retention. Dietary inclusion of DDGS tended to decrease production cost (€ per kg of fish), and dietary NAT supplementation further reduced production cost, which was significantly lower with diet DDGS35ENZ than with the control. Whole-body composition, hepatosomatic and visceral indexes were not affected by the dietary inclusion of DDGS, though a trend was noticed for a decrease of whole-body lipid, energy, and visceral index with the increase dietary DDGS. Plasma glucose, protein, albumin, and globulins levels were similar among diets, whereas plasma triglycerides increased, and cholesterol decreased with the increase of dietary DDGS. Hepatic glycolytic enzymes activities (hexokinase and glucokinase) were similar among groups, while gluconeogenic (fructose biphosphatase) activity, GDH and ASAT activities decreased with the increase of dietary DDGS. The hepatic activity of oxidative stress-related enzymes catalase and superoxide dismutase were not affected by dietary treatments, but G6PDH and GR activities decreased, and liver lipid peroxidation increased with dietary DDGS level. Dietary NAT supplementation decreased overall lipid peroxidation.

Overall, results of this study indicate that total replacement of SBM by DDGS in diets for gilthead seabream did not compromise growth performance or feed utilization efficiency, while supplementation of DDGS-based diet with exogenous carbohydrases improved feed utilization efficiency and economic efficiency ratio.

Keywords: by-products, growth performance, intermediary metabolism, nutrient utilization, oxidative stress.

1. Introduction

Driven by economic and environmental issues, the search for alternatives to fisheries ingredients use in aquafeeds has been a priority to ensure aquaculture sustainability. Several alternative ingredients have been considered, mainly plant ingredients, being soybean meal (SBM) the most studied and used in aquafeeds (Gatlin et al., 2007; Welker et al., 2014). However, SBM and other traditionally plant ingredients used in aquafeeds also have some constraints, as they are becoming increasingly important for human consumption and are available in the international market at high pieces (Matos et al., 2017).

Agro-industrial by-products may have an important role to address the sustainability challenges of aquaculture, being an alternative to traditional fisheries and plant ingredients (Ajila et al., 2012). In recent years, an increase of ethanol production from cereals (corn, wheat, sorghum, etc.) led to an increased availability of dried distiller's grains with solubles (DDGS), the major by-product of dry cereal milling from fuel ethanol production. DDGS is now readily available, including in EU, and is considered an economically competitive source of energy and protein in world feed market (Lim et al., 2011; Welker et al., 2014). Moreover, nutrient concentration in DDGS is higher than in the original cereals. For instance, corn DDGS contains three times more nutrients than corn. Corn DDGS protein content ranges from 26 to 33%, lipids from 9 to 14%, and neutral detergent fibers from 33 to 44%. Moreover, excluding non-starch polysaccharides (NSP) it does not contain other antinutritional factors, as those found in other plant ingredients (Liu, 2011; Welker et al., 2012).

DDGS is now broadly used in farm animal feeds but not yet in aquafeeds. However, increased scientific evidence highlighted its potential as an aquafeed ingredient, particularly to omnivorous fish. For example, in channel catfish, Ictalurus punctatus, first studies demonstrated that DDGS could be incorporated up to 40% or 70% in non-supplemented or lysine supplemented diets, or up to 90% in winter diets (Tidwell et al., 1990; Webster et al., 1991; Webster and Tidwell, 1992). More recent studies have however recommended lower optimal dietary inclusion levels, of circa 30 to 35% (Robinson and Li, 2008; Lim et al., 2009; Zhou et al., 2010; Renukdas et al., 2014). A maximum dietary DDGS inclusion level of 33% was established for hybrid striped bass, Morone chrysops × M. saxatilis (Trushenski and Gause, 2013). Relatively higher dietary DDGS inclusion levels, up to 50-60%, can be incorporated in diets for Nile tilapia, Oreochromis niloticus, if supplemented with lysine (Wu et al., 1996, 1997; Shelby et al., 2008; Abo-State et al., 2009). In rainbow trout, a carnivorous species, DDGS may replace fish meal up to 10% (Stone et al., 2005; Barnes et al., 2012) or 22% (Cheng and

Hardy, 2004) in lysine and methionine supplemented diets. Also, in rainbow trout, it was shown that DDGS can be incorporated in the diets up to 50%, replacing plant feedstuffs mixture (sunflower meal, rapeseed meal, and field peas) (Øverland et al., 2013a). However, for turbot, Scophthalmus maximus, another carnivorous species, dietary inclusion of 10% of DDGS replacing FM reduced growth performance (Diógenes, et al., 2018a).

The lower performance of carnivorous fish fed diets including DDGS has been attributed to the high level of NSP of DDGS, which may impair nutrient utilization (Diógenes, et al., 2018a, b). Supplementation of aquafeeds with exogenous carbohydrases to improve NSP digestibility is recent but seems to have great potential (Castillo and Gatlin, 2015). Indeed, it was shown that exogenous carbohydrases increased digestibility of DDGS-based diets for turbot (Diógenes et al., 2018b).

The aim of the present study was to evaluate the effect of dietary replacement of SBM by DDGS and of exogenous carbohydrases supplementation on growth performance, production costs, and hepatic oxidative status of gilthead sea bream, Sparus aurata, an important carnivorous fish species in the Mediterranean.

2. Materials and Methods

The study was performed at the Marine Zoological Station, University of Porto, Portugal, by certified scientists (following the Federation of European Laboratory Animal Science Associations - FELASA category C recommendations), according to the European Economic Community animal experimentation guidelines directive of 24 November 1986 (86/609/EEC).

2.1 Diets

Four diets were formulated to be isoproteic (47% crude protein) and isolipidic (18% crude lipids). The control diet (diet DDGS0) was formulated with 35% of FM and a mixture of plant ingredients. Two other diets were formulated incorporating 15 or 35% of DDGS, replacing 37.5% or 100% of SBM of the control diet (diets DDGS15 and DDGS35, respectively). Another diet was formulated similar to diet DDGS35, but including a commercial exogenous enzyme complex, Natugrain®TS, BASF (NAT; diet DDGS35ENZ). NAT is a complex of highly purified non-starch polysaccharides enzymes, namely endo-1,4-beta-xylanase (5600 TXU/g) and endo-1,4-beta-glucanase (2500 TGU/g). NAT was incorporated in the diet before pelleting, as recommended for pelleted feed up to 85°C (BASF recommendations). Diets were supplemented with methionine

and lysine to meet the EAA requirements estimated for the species (Peres and Oliva-Teles, 2009). All ingredients were finely ground, thoroughly mixed, and dry pelleted using a laboratory pellet mill (CPM: California Pellet Mill, Crawfordsville, IN, USA) through a 3 mm die. Diets were dried in an oven for 24 h at 40 °C and then stored at -20 °C until used. The formulation and proximate composition of the diets are presented in Table 1.

2.2 Growth trial

The growth trial was performed in a thermo-regulated recirculating water system, equipped with a battery of 12 fiberglass cylindrical tanks of 300L water capacity, supplied with a continuous flow of filtered seawater (flow rate increased throughout the trial from 4 to 6 L min-1). Gilthead seabream (Sparus aurata) juveniles were provided by a commercial fish farm and kept in quarantine for one month. Then, fish were transferred to the experimental systems and adapted to the experimental conditions for 15 days. During this period, fish were fed a commercial diet (48% protein and 17% lipids; Sorgal S.A., Ovar, Portugal). At the beginning of the trial, 12 homogenous groups of 20 fish (initial body weight 15 ± 1 g) were randomly distributed into the tanks, and each diet was randomly assigned to triplicate of these groups. Fish were fed by hand, twice a day, at 9.30 h and 16.30 h, 6 days a week, for 67 days. Utmost care was taken to avoid feed waste and to assure that all feed supplied was consumed. Fish were bulk weighted at the beginning, after four weeks, and at the end of the trial, following one day of feed deprivation. Throughout the experimental period temperature was regulated to 24 ± 1°C, salinity averaged 35.5 ± 0.8 ‰, oxygen level averaged 7.0 mg L-1, and photoperiod was adjusted to 12 h light as 12 h dark.

At the beginning of the trial 10 fish were sampled from the stock population and at the end of the trial four fish were sampled from each tank, pooled, and frozen for whole-body composition analysis. Whole-fish, viscera and liver weights were recorded for determination of visceral (VI) and hepatosomatic (HSI) indices. To minimize manipulation stress, the remaining fish continued to be fed for three more days, and then blood and liver from three fish per tank were randomly sampled 4 h after the morning meal. Blood was sampled from the caudal vein with a heparinized syringe, immediately centrifuged and the plasma frozen at −20 °C until analysis. Fish were then euthanized with a sharp blow to the head and immediately eviscerated in ice-cooled tray. The liver was excised, immediately frozen in liquid nitrogen, and stored at −80 °C until measurement of enzymes activities.

Table 1
Formulation and proximate composition (% dry matter) of the experimental diets.

Diet	DDGS0	DDGS15	DDGS35	DDGS35ENZ
Ingredients				
Fish meal[1]	35	35	35	35
CPSP[2]	1	1	1	1
DDGS[3]	–	15	35	35
Soybean meal[5]	20	12.5	–	–
Wheat gluten[4]	7.4	7.8	9.5	9.5
Wheat meal[6]	18.7	11.8	3.8	3.7
Fish oil	13.9	13.0	11.8	11.8
Vitamin premix[7]	1	1	1	1
Choline chloride (50%)	0.5	0.5	0.5	0.5
Mineral premix[8]	1	1	1	1
Binder[9]	1	1	1	1
Taurine[10]	0.3	0.3	0.3	0.3
Lysine[10]	–	–	0.12	0.12
Methionine	0.24	0.08	–	–
Natugrain®TS[11]	–	–	–	0.1
Proximate and amino acid composition (% dry matter)				
Dry matter (%)	91.8	94.5	95.0	95.1
Crude protein	47.8	47.2	47.5	47.5
Crude lipid	18.4	18.4	18.9	19.0
Starch	11.3	7.5	3.8	3.7
Energy (kJ g^{-1})	22.4	21.9	22.5	22.4
Ash	9.4	9.8	9.5	10.3
Lysine	2.79	2.67	2.61	–
Arginine	2.77	2.61	2.43	–
Histidine	1.54	1.42	1.39	–
Isoleucine	2.07	2.01	1.83	–
Leucine	3.48	3.54	3.85	–
Valine	2.88	2.74	2.75	–
Methionine	1.23	1.26	1.36	–
Cysteine	0.80	0.95	0.99	–
Phenylalanine	2.00	1.82	1.61	–
Tyrosine	2.83	2.73	2.67	–
Threonine	2.12	1.95	2.24	–
Aspartic Acid	4.50	4.33	4.02	–
Glutamic Acid	6.27	6.21	6.25	–
Serine	1.82	1.81	1.80	–
Glycine	2.84	2.94	2.91	–
Alanine	3.96	4.23	4.31	–
Proline	2.49	2.58	2.69	–
Taurine	0.40	0.41	0.39	–

[1]Pesquera Centinela, Steam Dried LT, Chile (CP: 69.7%; CL 7.2%). Sorgal, S.A. Ovar, Portugal.
[2]Soluble fish protein concentrate (CP: 80.4% DM; GL: 19.7% DM Sopropêche, France.
[3]DDGS (CP: 32.8%; CL:9.0%; Starch: 0.5%) Pannonia Gold®.
[4]Wheat gluten (CP: 86.2%; CL: 3.0%), Sorgal, S.A. Ovar, Portugal.
[5]Soybean meal (CP: 51.9%; CL:3.7%), Sorgal, S.A. Ovar, Portugal.
[6]Wheat meal (CP: 14.5%; CL:2.4%), Sorgal, S.A. Ovar, Portugal.
[7]Vitamins (mg kg^{-1} diet): retinol, 18000 (IU kg^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.
[8]Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.4 (g kg^{-1} diet).
[9]Aquacube. Agil, UK.
[10]Feed grad amino acids, Sorgal, S.A. Ovar, Portugal. [11]Natrugain®TS, BASF, Germany.

2.3 Analytical methods

2.3.1 Proximate analysis

Proximate analysis of the ingredients, diets, and whole-fish was made by the following procedures: dry matter, by drying the samples at 105°C until constant weight; protein content (N x 6.25) by the Kjeldahl method following acid digestion, using Kjeltec digestion and distillation units (Tecator Systems, Höganäs, Sweden; models 1015 and 1026, respectively); lipid content by extraction with petroleum ether using a Soxtec system (Tecator Systems, Höganäs, Sweden; extraction unit model 1043 and service unit model 1046); ash by incineration in a muffle furnace at 450 °C for 16 h, starch according to Beutler (1984); and energy by direct combustion in an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261). For amino acid analysis, diet samples were hydrolyzed for 23 h with 6M HCl at 110 °C under nitrogen atmosphere and derivatized with phenylisothiocyanate (PITC; Pierce) reagent before separation by high performance liquid chromatography (HPLC) in a Waters Reversed-Phase Amino Acid Analysis System (Waters auto sample model 717 plus; Waters binary pump model 1525; Waters dual absorbance detector model 2487), equipped with a PicoTag column. External standards (Pierce NC10180) were prepared along with the samples, and norleucine was used as internal standard. Tryptophan was not determined.

2.3.2 Plasma analysis

Plasma metabolites were analyzed using commercial kits from Spinreact, S.A. (Gerona, Spain): glucose (ref: 1001191), total protein (ref: 1001291), triglycerides (TAG; ref: 1001312), total cholesterol (ref: 1001090), and albumin (ref: 1001020). Globulins were determined as the difference between total protein and albumin.

2.4 Enzyme activity

Liver was homogenized in ice-cold buffer (100mM-Tris-HCl, 0.1mM-EDTA and 0.1 % triton X-100 (v/v), pH 7.8) and centrifuged at 30 000g for 30 min at 4 °C. The resultant supernatants were collected, and aliquots stored at -80°C until enzyme activity analysis. All enzyme activities were measured at 37 °C in a microplate reader (ELx808™; BioTek Instruments), by monitoring changes in absorbance.

2.4.1 Intermediary metabolism enzymes activities

Glutamate dehydrogenase (GDH; EC 1.4.1.2) activity was performed using a reaction mixture containing 50 mM imidazole–HCl buffer (pH 7.4), 0.2 mM NADH, 1 mM

ADP, 100 mM ammonium acetate, 2 units mL−1 LDH and 10 mM α-ketoglutarate (Morales et al., 1990). Aspartate aminotransferase (ASAT; EC 2.6.1.1) activity was determined as described by Singer et al. (1990) and the reaction mixture contained 50 mM imidazole-HCl buffer (pH 7.4), 10 mM α-ketoglutarate, 0.3 mM NADH, 0.05 mM pyridoxal phosphate, 3 units mL−1 MDH and 25 mM L-aspartate. Alanine aminotransferase (ALAT; EC 2.6.1.2) activity was determined as described by Morales et al. (1990) and the reaction mixture contained 50 mM imidazole-HCl buffer (pH 7.4), 10 mM α-ketoglutarate, 0.2 mM NADH, 0.05 mM pyridoxal phosphate, 2 units mL−1 LDH and 25 mM L-alanine. Hexokinase (HK; EC 2.7.1.1) and glucokinase (GK; EC 2.7.1.2) activities were determined as described by Vijayan et al. (1990), and the reaction mixture contained 50 mM imidazole-HCl buffer (pH 7.4), 2.5mMATP, 5mMMgCl2, 0.4mMNADP, 2 units mL−1 G6PDH and 1 mM (HK) or 100 mM (HK-IV) glucose. Pyruvate kinase (PK; EC 2.7.1.40) activity was performed with a reaction mixture consisting of 50 mM imidazole-HCl buffer (pH 7.4), 5 mM MgCl2, 100 mM KCl, 0.15 mM NADH, 1 mM ADP, 2 units mL−1 LDH and 2 mM PEP (Morales et al., 1990). Fructose 1,6-bisphosphatase (FBPase; EC 3.1.3.11) activity was performed with a reaction mixture consisting of 50 mM imidazole-HCl buffer (pH 7.4), 5 mM MgCl2, 12 mM 2-mercaptoethanol, 0.5 mM NADP, 2 units mL−1 G6PDH, 2 units mL−1 PGI and 0.5mMfructose 1,6- bisphosphate (Morales et al., 1990). All enzyme activities are expressed as milliunits per milligram of soluble protein (specific activity). Protein concentration was determined by Bradford's method (1976), with bovine serum albumin solution as standard.

2.4.2 Antioxidant enzymes activities

Glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity was measured as described by Morales et al. (1990), using a reaction mixture containing 50 mM imidazole-HCl buffer (pH 7.4), 5 mM MgCl2, 2 mM NADP and 1 mM glucose-6-phosphate. Glutathione reductase (GR; EC 1.6.4.2) activity was determined at 340 nm by measuring the oxidation of NADPH as described by Morales et al. (2004). The reaction mixture consisted of 0.1 M-sodium phosphate buffer (pH 7.5, Sigma), 1 mM-EDTA, 0.63 mM-NADPH (Sigma) and 0.16 mM-GSSG (Sigma). Catalase (CAT; EC 1.11.1.6) activity was determined according to Aebi (1984) by measuring the decrease in H2O2 concentration at 240 nm. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7, Sigma) and 10 mM-H2O2 (Sigma) freshly added. Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured at 550 nm by the ferricytochrome C method, using xanthine/xanthine oxidase as the source of superoxide radicals (McCord and Fridovich, 1969). The reaction mixture consisted of 50 mM-potassium

phosphate buffer (pH 7.8 Sigma), 0.1 mM-EDTA (Sigma), 0.1 mM-xanthine (Sigma), 0·012 mM-cytochrome C (Sigma) and 0.025 IU/mL xanthine oxidase (Sigma). SOD activity is expressed as units per mg of protein. One unit of activity is defined as the amount of enzyme necessary to produce 50% inhibition of ferricytochrome C reduction rate. All other enzyme activities are expressed as units (CAT) or milliunits (GR; G6PDH) per mg of soluble protein. One unit of enzyme activity was defined as the amount of enzyme required to transform 1 µmol of substrate/min under the above assay conditions.

2.5 Lipid peroxidation

Hepatic lipid peroxidation (LPO) levels were determined based on the concentration of malondialdehyde (MDA) as described by Buege and Aust (1978). An aliquot of supernatant from the homogenate (100 µL) was mixed with 500 µL of a previously prepared solution containing 15% (w/v) TCA (Sigma), 0.375% (w/v) thiobarbituric acid (Sigma), 80% (v/v) HCl 0.25 N and 0.01% (w/v) butylated hydroxytoluene (Sigma). The mixture was heated to 100 °C for 15 min and, after being cooled to room temperature and centrifuged at 1500g for 10 min, the absorbance was measured at 535 nm in the supernatant. MDA concentration is expressed as nmol MDA per g of wet tissue, calculated from a calibration curve.

2.6 Statistical analysis

Data were checked for normal distribution and homogeneity of variances and when necessary normalized (log or arcsine square root transformation). The effect of the dietary treatments on the different parameters was analyzed by one-way ANOVA. Significant differences among means ($P<0.05$) were determined by the Tukey's multiple range test. All statistical analyses were carried out using the SPSS 25.0 software package for Mac.

3. Results

Fish accepted well all diets and mortality during the trial was very low and not affected by dietary treatments. At the end of the trial, mean body weight more than quintupled in all treatments and there were no differences in growth performance between groups (Table 2). Feed intake was not affected by the dietary inclusion of DDGS, but it was lower in fish fed the NAT supplemented diet than in the control. Similarly, feed and protein efficiency ratio as well as nitrogen retention were not affected by dietary DDGS incorporation but, compared to the control, improved by dietary NAT

supplementation. Energy retention was higher with the NAT supplemented diet than with diet DDGS35. Feeding cost per day and per kg of body weight gain (€ day-1 or € kg fish-1) tended to be lower with DDGS diets and were significantly lower with the NAT supplemented diet than with the control.

Table 2
Growth performance, feed efficiencies and economic indices of gilthead seabream juveniles fed the experimental diets.

	DDGS0	DDGS15	DDGS35	DDGS35ENZ	SEM
Fish					
Initial body weight, IBW (g)	15.0	15.0	15.0	15.0	0.00
Final body weight, FBW (g)	79.3	82.0	76.5	77.9	0.92
Weight gain (g kg ABW*$^{-1}$ day^{-1})	20.3	20.6	20.0	20.2	0.09
Daily growth index[1]	2.73	2.80	2.65	2.69	0.02
Feed intake (g/kg/day)	28.4^{b}	26.7^{ab}	27.1^{ab}	22.5^{a}	0.82
Feed efficiency[2]	0.72^{a}	0.77^{ab}	0.73^{a}	0.90^{b}	0.03
Protein efficiency ratio[3]	1.51^{a}	1.63^{ab}	1.54^{ab}	1.89^{b}	0.06
Mortality (%)	0.00	1.67	1.67	0.00	0.56
Nitrogen retention (% N intake)[5]	26.5^{a}	28.1^{ab}	27.3^{ab}	34.1^{b}	1.09
Energy retention (% E intake)[5]	32.7	34.1	29.6	36.5*	1.14
Economic indices					
Diet price¥	1	0.988	0.978	0.988	
Feeding cost (€ day^{-1})[6]	28.4^{b}	26.4^{ab}	25.9^{ab}	22.3^{a}	0.8
Feeding cost (€ kg fish^{-1})[7]	1.40^{b}	1.30^{ab}	1.32^{ab}	1.10^{a}	0.04

Values presented as means (n = 3) and pooled standard error of the mean (SEM). Means in the same row with superscript letters are significantly different (P<0.05).
*denotes significant differences between DDGS35 and DDGS35ENZ diets (*t*-test; P<0.05).
¥Price of the diets calculated on the basis of the relative value of the control diet, assuming to be 1.
ABW: Average body weight: (initial body weight + final body weight)/ 2.
[1]DGI = ((FBW$^{1/3}$ – IBW$^{1/3}$)/ time in days) × 100.
[2]FE = wet weight gain / dry feed intake.
[3]PER = wet weight gain / crude protein intake
[4]Nitrogen or energy retention (g or kJ kg ABW^{t-1} day^{-1}) = FBW × FBN − IBW × IBN)/(((IBW + FBW)/2) × days)
[5]Nitrogen retention = (FBW × FBN − IBW × IBN) / (NI)) × 100
[6]Feeding cost (€ day^{-1}) = feed intake × diet price
[7]Feeding cost (€ kg fish^{-1})^{7} = Feed efficiency / diet price

At the end of the trial, whole-body dry matter, protein, lipid, ash, and energy contents as well as HSI and VI did not differ among dietary treatments (Table 3).

Table 3
Whole-body composition of gilthead seabream juveniles fed the experimental diets.

	Initial	DDGS0	DDGS15	DDGS35	DDGS35ENZ	SEM
Whole-body composition (% wet weight)						
Dry matter (%)	25.6	34.0	33.7	32.9	32.9	0.21
Protein	13.4	16.8	16.6	16.9	17.0	0.09
Lipids	7.76	13.4	13.4	12.0	12.2	0.32
Ash	5.7	3.9	4.0	3.8	3.7	0.05
Energy (kJ kg^{-1})	5.5	9.3	9.0	8.3	8.4	0.17
Relative tissue weight (%)						
Hepatosomatic index[1]	–	1.11	1.28	1.18	1.11	0.03
Visceral index[2]	–	8.18	8.11	7.79	7.41	0.15

Values presented as means (n = 3) and pooled standard error of the mean (SEM). Means in the same row with superscript letters are significantly different (P<0.05).
[1]HSI = (liver weight/body weight) × 100.
[2]VI = (viscera weight/body weight) × 100.

Dietary DDGS or NAT supplementation did not affect plasma glucose, total protein, albumin, and globulins levels, while plasma triglycerides increased with dietary DDGS inclusion (Table 4). Plasma cholesterol tended to decrease with dietary DDGS inclusion, and it was significantly lower in fish fed the NAT supplemented diet than the control diet.

Table 4
Plasma metabolites concentration (mg dL^{-1}) of gilthead seabream juveniles fed the experimental diets.

	DDGS0	DDGS15	DDGS35	DDGS35ENZ	SEM
Glucose	95.6	100.0	85.9	103.9	2.77
Total Protein	4.37	4.33	4.48	4.64	0.07
Albumin	1.07	1.04	1.04	1.00	0.02
Globulins	3.30	3.29	3.44	3.64	0.07
Triglycerides	294.4^{a}	319.1^{a}	460.8^{b}	451.6^{b}	19.09
Cholesterol	322.4^{b}	320.0^{ab}	274.4^{ab}	270.1^{a}	7.70

Values presented as means (n = 9) and pooled standard error of the mean (SEM). Means in the same row with superscript letters are significantly different ($P<0.05$).

Specific activity of hepatic key enzymes of glycolysis was not affected by dietary treatments while activity of the gluconeogenic enzyme FBPase decreased with the increase of dietary DDGS (Table 5). GDH and ASAT activities also decreased with the increase of dietary DDGS. Dietary NAT supplementation did not affect the activity of the intermediary metabolism enzymes analyzed.

Table 5
Specific activities of liver enzymes involved in key intermediary metabolism of gilthead seabream fed the experimental diets. Data are expressed as mU mg^{-1} protein.

	DDGS0	DDGS15	DDGS35	DDGS35ENZ	SEM
Amino acid catabolism					
Glutamate dehydrogenase	346.1^{b}	338.0^{ab}	267.5^{a}	338.9^{ab}	11.40
Alanine aminotransferase	143.7	146.9	125.1	145.7	3.27
Aspartate aminotransferase	644.9^{b}	643.1^{b}	458.2^{a}	579.1^{a}	22.62
Glycolysis					
Hexokinase	4.94	4.90	4.05	4.22	0.17
Glucokinase	5.92	5.79	5.74	6.70	0.30
Pyruvate kinase	16.0	14.4	12.0	14.1	0.61
Gluconeogenesis					
Fructose-1,6-bisphosphatase	29.0^{b}	24.0^{ab}	19.3^{a}	23.2^{a}	0.96

Values presented as means (n = 9) and pooled standard error of the mean (SEM). Means in the same row with superscript letters are significantly different ($P<0.05$).

Specific activity of oxidative stress-related enzymes tended to decrease with dietary DDGS supplementation, and this effect was statistically significant for G6PDH and GR (Table 6). LPO levels increased with the increase of dietary DDGS, while it significantly decreased with dietary NAT supplementation. Dietary NAT supplementation also increased GR activity.

Table 6
Specific activities of liver oxidative stress pathways and hepatic lipid oxidation (LPO) of gilthead seabream fed the experimental diets. Data are expressed as mU mg^{-1} protein.

	DDGS0	DDGS15	DDGS35	DDGS35ENZ	SEM
Glucose-6-phosphate dehydrogenase	200.3^{c}	149.7^{b}	93.2^{a}	99.1^{a}	10.51
Glutathione reductase	5.36^{b}	5.46^{b}	3.66^{a}	5.09^{b}	0.22
Catalase	76.2	73.2	61.5	80.4	2.82
Superoxide dismutase (U mg^{-1} protein)	302.3	309.6	269.1	259.1	11.19
Lipid peroxidation (nmol MDA g^{-1} tissue)	8.37^{ab}	10.8^{ab}	12.2^{b}	7.57^{a}	0.65

Values presented as means (n = 9) and pooled standard error of the mean (SEM). Means in the same row with superscript letters are significantly different ($P<0.05$).

4. Discussion

To the best of our knowledge, the potential use of DDGS in gilthead seabream diets has not been yet addressed. In the present study DDGS was used to replace soybean meal at a constant FM level, thus avoiding confounding effects due to FM levels, as suggested by Øverland et al. (2013a). Results indicate that DDGS can be incorporated up to 35% in the diets for gilthead sea bream juveniles, totally replacing SBM, without affecting growth performance, feed intake and feed utilization efficiency. Moreover, replacing SBM by DDGS allowed a reduction of feeding cost per day of circa 9% and per unit of body weight gain of circa 6%.

The maximum dietary DDGS level used in the present study is higher than that considered acceptable for other carnivorous fish. Depending on species and dietary ingredients used, DDGS may be incorporated around 10-40%. For carnivorous fish, maximum dietary DDGS inclusion was estimated to be 10-20% for rainbow trout (Welker et al., 2014), 14-21% for olive flounder, Paralichthys olivaceus (Rahman et al., 2015), and less than 10% for turbot (Diogenes et al., 2018b). Nevertheless, high DDGS incorporation levels are tolerated by omnivorous species (Li et al., 2010, 2011a; Lim et al., 2011. Schaeffer et al., 2011; Zhou et al., 2010).

Dietary inclusion of DDGS may be limited by its relatively lower level of some EAA, relatively to FM and SBM, being lysine the most limiting EAA. For example, it was observed that FM replacement by DDGS can be increased from 15 to 22.5% in rainbow trout and from 30 to 40% in tilapia if the diets were supplemented with lysine (Cheng and

Hardy, 2004; Li et al., 2011a). Relatively to the ideal EAA profile of gilthead seabream (Peres and Oliva-Teles, 2009), DDGS was deficient in lysine and therefore DDGS-based diets had to be dully supplemented with crystalline lysine to restore its EAA profile. On the contrary, DDGS proved to be a good source of methionine.

The indigestible NSP content of DDGS is two to three times higher than that of the original grain, and this can also potentially limit DDGS inclusion in diets, especially for carnivorous fish, which are less tolerant to dietary fiber than omnivorous or herbivorous fish. High dietary NSP content may impair overall digestive nutrient uptake by increasing chime viscosity, which obstructs digestive enzymes action and microvilli absorption capability, and also affects chime movement along the digestive tract (Sinha et al., 2011; Castillo and Gatlin, 2015). Exogenous enzymes that hydrolyze NSP may be used to overcome this limitation; however, results of their use in fish diets are still inconsistent (Castillo and Gatlin, 2015). Excluding exogenous phytase, which efficacy to improve phosphorus digestibility is well documented, the use of carbohydrases has been lagged considerably (revised by Castillo and Gatlin, 2015). Previous studies using plant-based diets supplemented with NAT have shown that dry matter and protein digestibility improved in European seabass bass, Dicentrarchus labrax (Diaz-Rosales et al., 2014), while no effects were observed for silver perch, Bidyanus bidyanus (Stone et al., 2003) and white seabass, Diplodus sargus (Magalhães et al., 2016). Recently, it was also shown that NAT supplementation improved dry matter, protein, lipids, some amino acids, and energy digestibility of DDGS-based diets in turbot juveniles (Diogenes et al., 2018b). In the present study, diet digestibility was not evaluated, but the higher feed efficiency observed with NAT supplementation suggest that it effectively improved diet digestibility. Moreover, feed intake was also lower in fish fed the NAT supplemented diet. As feed intake is usually regulated by digestible energy intake (Cho and Kaushik, 1985; Peres et al., 2011) the lower feed intake can at least in part be related to higher available energy provided by the NAT supplemented diet.

Whole-body composition, hepatosomatic and visceral indices were not affected by dietary replacement of SBM by DDGS or by diet supplementation with NAT. For other species it has been shown that high dietary DDGS inclusion levels reduced whole-body lipids and energy content (Webster et al. 1991; Robinson and Li 2008; Li et al., 2011b) that was attributed to a reduction of digestible energy intake (Diógenes et al., 2018a), which was not the case of present study.

Plasma metabolites analyzed are within normal values for gilthead sea bream (Peres et al., 2013) and were not affected by diet composition, except for triglycerides that increased and cholesterol that decreased with dietary inclusion of DDGS replacing

SBM. Previously, it was observed in turbot that FM replacement by DDGS reduced both plasma triglycerides and cholesterol levels, and the authors attributed this reduction to lower feed intake of fish fed DDGS diets (Diogenes et al., 2018a), which was not observed in the present study. Differences in fatty acids profile among diets may contribute in part to explain the differences observed in plasma cholesterol and triglycerides levels. As DDGS had higher lipid content than SBM, dietary inclusion of DDGS led to a reduction of fish oil content to keep diets isolipidic. It is known that dietary replacement of fish oil by vegetable oils affects plasma cholesterol and triglycerides levels of gilthead seabream (Caballero et al., 2006; Castro et al., 2016) and other marine fish species (Richard et al., 2006; Torstensen et al., 2011; Bowyer et al., 2012), and an analogous effect might have occurred with DDGS oil. Moreover, the presence of yeast cells in DDGS (approximately 4%; Ingledew, 1999) may have also contributed to these results. Indeed, it was reported that diet supplementation with yeast or yeast-derived products may affect plasma lipid profile, decreasing plasma cholesterol (Kumar et al., 2013; Øverland et al., 2013b) or, in opposition, increasing plasma cholesterol and decreasing triglycerides (Mohebbi et al., 2013). Further studies are therefore needed to elucidate the effect of DDGS oil on plasma lipid profile in marine fish species.

The hepatic activity of amino acid catabolic enzymes (GDH and ASAT) decreased with the dietary inclusion of DDGS, suggesting a protein sparing effect of SBM replacement by DDGS, which, however, was not confirmed by the whole-body nitrogen retention (% nitrogen intake) data. The FBPase activity was also reduced with the increase of dietary DDGS inclusion, indicating a decrease of gluconeogenesis. Taken together, these results seem to indicate that a lower amount of excess amino acids were available for energy purposes in the DDGS supplemented diets. Similarly, the reduction of dietary protein uses for energetic purposes has been correlated with the reduction of amino acid catabolic enzymes activity (Gomez-Requeni et al., 2003; Peres and Oliva-Teles, 2007; Caballero-Solares et al., 2015), even though other authors did not observe such effect (Gomez-Requeni et al., 2004; Guerreiro et al., 2014; Coutinho et al., 2016).

Though it is assumed that NAT improved NSP digestibility, thus potentially increasing available glucose for energy purposes, fish fed the NAT supplemented diet eat less and thus overall available glucose might not have increased. This may explain why glycolysis did not increase in fish fed the NAT supplemented diet. NAT supplementation also lead to an increase the activity of GDH and FBPase activities tended to increase in fish fed the NAT supplemented diet, indicating an increased glucose synthesis from glycogenic amino acids. This suggests that NAT supplemented

diet provided an excess of available amino acids in comparison to the non-supplemented diet.

Diet has a critical role in the physiological balance between reactive oxygen species (ROS) and antioxidant defenses. Diet may induce a shift towards an increase of ROS levels or a decrease of antioxidant defense, inducing DNA damage, and lipid and protein oxidation. Glutathione redox system is the primary enzymatic system ensuring the detoxification of ROS by oxidation of reduced glutathione into glutathione-disulfide, which is then recycled by GR. The NADPH required for this process is mainly provided by the pentose phosphate pathway through the action of G6PDH, one of the rate-limiting enzymes. In the present study, dietary incorporation of DDGS reduced G6PDH and GR activities, and the activities of CAT and SOD also tended to be reduced, though this reduction was not statistically significant. This overall reduction in the activity of counter-acting oxidative stress enzymes led to an increased liver lipid peroxidation. Interestingly, NAT supplementation counteracted the increased susceptibility to hepatic oxidative stress induced by the dietary inclusion of DDGS. The cause of these results is not clear, and several hypotheses may be equated as the enhanced production of microbial short-chain fatty acids, the end products of dietary NSP fermentation, with antioxidant proprieties (Enes et al., 2012).

A profitability analysis of replacing dietary feedstuffs or of using exogenous enzymes is essential to evaluate the economic return of diet modifications (Bedford and Schulze, 1998) but has been little studied in fish. This study indicates that replacing SBM by DDGS slightly reduced diet price and reduced feeding costs per kg of fish produced by 5.7%. More impressive was the effect of dietary supplementation with NAT, which reduced feeding costs per kg of fish produced by 16.6% comparatively to diet DDGS35. Together, dietary inclusion of DDGS and NAT supplementation significantly reduced feeding costs per kg of fish produced by 21.4%.

Overall, results of this study indicate that total replacement of soybean meal by DDGS in diets for gilthead sea bream juveniles does not compromise growth performance and feed utilization, while slightly improved feeding cost per kg of fish produced. Concomitant supplementation of DDGS diets with an exogenous carbohydrases complex significantly improved feed efficiency and reduced the feeding cost per kg of fish produced. Moreover, NAT supplementation totally counteracted the increased susceptibility to hepatic oxidative stress induced by the 35% DDGS.

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Chapter 5

Effects of dietary tryptophan and chronic stress in gilthead seabream (*Sparus aurata*) juveniles fed corn distillers dried grains with solubles (DDGS) based diets

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ABSTRACT

Distillers' dried grains with solubles (DDGS) has low tryptophan (Trp) relatively to the branched-chain amino acids (BCAA) levels, and this may reduce transport of Trp through the blood-brain barrier due to competition for the same transport carrier. This may affect synthesis and release of serotonin, with negative consequences in stress tolerance. In the present study, it is hypothesized that a Trp/BCAA unbalance in high DDGS diets may impair the capacity of gilthead seabream (*Sparus aurata*) juveniles to cope with chronic stress induced by high stocking density. Three DDGS-based diets (30%DDGS+13%FM) were formulated and supplemented with Trp at 0, 0.13, and 0.25% of the diet and tested in triplicate, at two initial stocking densities (5 and 16 kg m^{-3}), in a 2 × 3 total randomized factorial design. The growth trial was performed with 12g fish and lasted 64 days. Irrespective of the diet, high stocking density reduced growth performance and feed intake, but not feed efficiency. Plasma protein, triglycerides, and cholesterol levels; whole-body lipid, hepatosomatic index, and liver glycogen; hepatic activity of key-enzymes of glycolysis and lipogenesis were also reduced. Moreover, plasma glucose level and hepatic activity of key-enzymes gluconeogenesis were increased.

Irrespective of stocking density, diets supplementation with Trp did not affect growth and feed efficiency, but increased hepatic lipase activity and reduced liver lipids, plasma triglycerides and cholesterol levels, and hepatic activity of key-enzymes of amino acid catabolism. Moreover, dietary Trp supplementation restored plasma glucose levels of fish kept at high stocking density to levels similar to that of fish kept at low stocking density.

Overall, present results indicate that high stocking density reduced growth performance without affecting feed efficiency of gilthead seabream. Dietary Trp supplementation did not counteract the negative effect of stocking density on growth performance but seemed to mitigate stress response of gilthead seabream juveniles kept at high stocking density.

Keywords: chronic stress, plant feedstuffs, DDGS, gilthead seabream, stocking density, amino acids

1. Introduction

The heavy reliance of aquafeeds on fishmeal (FM) and fish oil (FO) as major protein and lipid sources is critical to the sustainable and economic development of aquaculture. The decreasing availability and increasing costs of FM have stimulated research on alternative protein sources, particularly plant proteins. However, the use of plant feedstuffs imposes some concerns due to the "food-feed competition", rising prices, and carbon footprint involved in their production and transport to the final consumers. Under this context, several plant and animal by-products have been studied, including distillers' dried grains with solubles (DDGS), a residual product from bio-ethanol production. DDGS is moderately high in protein and competitively priced relative to other alternative protein sources (Welker et al., 2014a). Nowadays, DDGS is successfully used in diets for different fish species, mainly omnivorous species as channel catfish, *Ictalurus punctatus* (Webster et al., 1991; Lim et al., 2009; Li et al., 2011a), rainbow trout, *Oncorhynchus mykiss* (Øverland et al., 2013; Welker et al., 2014a), Nile tilapia, *Oreochromis niloticus*, olive flounder, *Paralichthys olivaceus*, and hybrid tilapia, *Oreochromis niloticus* × *Oreochromis aureus* (Wu et al., 1997; Li et al., 2011b; Schaeffer et al., 2012; Welker et al., 2014b; Rahman et al., 2015; Bae et al., 2015). For carnivorous fish species as European seabass, *Dicentrarchus labrax*, meagre, *Argyrosomus regius*, and turbot, *Scophthalmus maximus*, the high fiber level and low dry matter and energy digestibility of DDGS are limiting its dietary incorporation (Magalhães et al., 2016; Diógenes et al., 2017). The unbalanced essential amino acids (EAA) profile of DDGS also deserves attention, namely the low tryptophan (Trp) to branched-chain amino acids (BCAA) ratio, as it may reduce brain Trp uptake, as both Trp and BCAA share the same blood-brain barrier transporter (Le Floc'h et al., 2011; Fernstrom, 2012).

Trp is required for protein synthesis and various metabolic functions, as insulin-like growth factor 1 (IGF-I) synthesis, regulation of appetite, glucose homeostasis, and stress, immune and inflammatory responses (Pérez-Sánchez and Le Bail, 1999; Le Floc'h and Seve 2007; Matte et al., 2011, Yao et al., 2011; Machado et al., 2015; Azeredo et al., 2017). Low Trp uptake into the brain may compromise serotonin synthesis, a neurotransmitter with relevant roles in behavioral and physiological responses, and that is biochemically derived from Trp. Indeed, dietary Trp supplementation has been reported to enhance fish brain serotonergic activity (Hseu et al., 2003; Papoutsoglou et al., 2005; Azeredo et al., 2017), with stress-mitigation (Lepage et al., 2002, 2003, 2005; Tejpal et al., 2014; Martins et al., 2013) and anti-inflammatory effects (Machado et al., 2015; Azeredo et al., 2017).

Therefore, the aim of the present study was to evaluate the effect of Trp supplementation of DDGS based diets for gilthead seabream (*Sparus aurata*) juveniles kept unstressed or under crowding stress conditions.

2. Materials and Methods

The experiment was carried out at the experimental facilities of the Marine Zoological Station, University of Porto, Portugal. Trained scientists (following FELASA category C recommendations) directed the trial and all procedures were conducted according to the recommendations of European Union Directive (2010/63/EU) on the protection of animals for scientific purposes.

2.1 Experimental diets

A low FM (13%DM) and high DDGS (30%DM) diet was formulated to meet the EAA requirements of gilthead seabream (Peres and Oliva-Teles, 2009) and was used as control (diet Trp1). Two other diets were formulated similar to the control but supplemented with Trp at 1.5 x (diet Trp1.5) or 2.0 x (Trp2) of the control diet level. Both feed grade Trp and taurine were coated with agar before mixing with the other ingredients. All ingredients were finely ground, thoroughly mixed, and pelleted using a laboratory pellet mill (CPM: California Pellet Mill, Crawfordsville, IN, USA) through a 2 mm die. Diets were dried in an oven at 40 °C for 24 h and then stored at -20º C until use. The formulation and proximate composition of the diets are presented in Table 1.

2.2 Growth trial

Gilthead seabream juveniles were obtained from a commercial fish farm and kept in quarantine for one month. Then, fish were transferred to the experimental systems and adapted to the experimental conditions for 15 days. During these periods, fish were fed a commercial diet (48% protein and 17% lipids; Sorgal S.A., Ovar, Portugal).

The growth trial was performed in two identical and independent thermo-regulated recirculating water systems, equipped with a battery of 9 fiberglass cylindrical tanks (45 L water capacity each), supplied with a continuous flow of filtered seawater. In each system, a 1.5 hp water pump (AstralPool, model Vitoria) pumped seawater that previously passed through a sump tank, a biofilter, a sand filter, and a 100 watts UV-light to each experimental tank at the rate of circa 6 L min^{-1}. An electric resistance placed in the sump tank regulated water temperature. Tanks and biofilter aeration was supplied with a blower and airstones. Throughout the trial, water quality parameters were

Table 1. Formulation and proximate composition (% dry matter) of the experimental diets.

Diet	Trp1	Trp1.5	Trp2
Ingredients			
Fish meal[1]	13	13	13
CPSP[2]	2	2	2
DDGS[3]	30	30	30
Corn gluten[4]	17.2	17	16.9
Soybean meal[5]	15	15	15
Wheat meal[6]	3.3	3.3	3.3
Fish oil	12.3	12.3	12.3
Agar	1	1	1
Vitamin premix[7]	1	1	1
Choline chloride (50%)	0.5	0.5	0.5
Mineral premix[8]	1	1	1
Binder[9]	1	1	1
Dicalcium phosphate	2.1	2.1	2.1
Taurine	0.5	0.5	0.5
Tryptophan	–	0.13	0.25
Proximate composition			
Dry matter (%)	90.0	91.3	90.6
Crude protein	43.7	43.7	43.8
Crude lipid	21.9	20.6	21.1
Ash	9.7	14.9	14.3
Tryptophan	**0.35**	**0.54**	**0.78**
Lysine	2.5	2.5	2.4
Arginine	2.8	2.8	2.7
Histidine	1.1	1.1	1.1
Isoleucine	1.9	1.9	1.9
Leucine	4.9	5.0	4.9
Valine	2.3	2.2	2.2
Methionine	1.2	1.2	1.1
Phenylalanine	2.6	2.6	2.6
Threonine	1.9	1.8	1.9
Tyrosine	2.9	2.9	2.9
Aspartic Acid	6.4	6.3	6.3
Glutamic Acid	2.7	2.8	2.7
Serine	1.6	1.5	1.6
Glycine	3.2	3.3	3.3
Alanine	3.6	3.5	3.6
Proline	2.5	2.5	2.4
SUM BCAA	9.1	9.1	9.0
TRP:BCAA ratio	0.038	0.059	0.087

[1]Pesquera Centinela, Steam Dried LT, Chile (CP: 74.2%; CL 10.1%). Sorgal, S.A. Ovar, Portugal.
[2]Soluble fish protein concentrate (CP: 80.4% DM; GL: 15.3% DM Sopropêche, France.
[3]DDGS (CP: 32.8%; CL:9.0%; Starch: 0.5%) Pannonia Gold®.
[4]Corn gluten (CP: 68.3%; CL: 2.9%), Sorgal, S.A. Ovar, Portugal.
[5]Soybean meal (CP: 53.7%; CL:2.1%), Sorgal, S.A. Ovar, Portugal.
[6]Wheat meal (CP: 14.6%; CL:2.2%), Sorgal, S.A. Ovar, Portugal.
[7]Vitamins (mg kg^{-1} diet): retinol, 18000 (IU kg^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.
[8]Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.4 (g kg^{-1} diet).
[9]Aquacube. Agil, UK.

monitored: temperature, salinity, and oxygen levels were checked daily and nitrogen compounds three times a week. Temperature averaged 24 °C, salinity averaged 35‰, dissolved oxygen averaged 7.0 mg L^{-1}, and ammonia and nitrites were kept below 0.02 l/min. Photoperiod was controlled to a 12L/12D.

At the beginning of the trial, fish had an average body weight of 12 g ± 2 g. To one experimental system were randomly distributed 9 homogeneous groups of 20 fish, and to the other experimental system were randomly distributed 9 homogeneous groups of 60 fish, corresponding to initial stocking densities of 5 and 16 kg m^{-3} (low and high stocking densities (LSD and HSD), respectively). The experimental diets were randomly assigned to triplicate groups at each density. The trial lasted 64 days, and during this period fish were fed twice a day, at 9.30 h and 16.30 h, to apparent satiation. Utmost care was taken to avoid feed losses.

2.3 Sampling

Fish in each tank were bulk-weighed at the beginning and at the end of the trial, after 1 day of feed deprivation. Ten fish from the initial stock population and 6 fish from each tank at the end of the trial were sampled, pooled for each tank, and frozen at -80°C for whole-body composition analysis. Whole fish, viscera, and liver weights were recorded for determination of hepatosomatic and visceral indices. To minimize manipulation stress, the remaining fish continued to be fed for 3 more days, after which 3 fish per tank were randomly sampled 4 h after the morning meal, to ensure that the intestine was full at sampling time. Fish were euthanized with a sharp blow to the head and immediately eviscerated in an ice-cooled tray. Liver was excised, immediately frozen in liquid nitrogen, and then stored at −80 °C until measurement of intermediary enzymes activity. Intestine was also excised and, after carefully removing adherent adipose and connective tissues, immediately frozen in liquid nitrogen and then stored at -80°C until measurement of digestive enzymes activity.

2.4 Analytical methods

2.4.1 Proximate analysis

Chemical analysis of ingredients, diets, and whole body were conducted as follows: dry matter, by drying the samples at 105°C until constant weight; protein content (N x 6.25) by the Kjeldahl method following acid digestion, using Kjeltec digestion and distillation units (Tecator Systems, Höganäs, Sweden; models 1015 and 1026, respectively); lipid content by extraction with petroleum ether using a Soxtec system

(Tecator Systems, Höganäs, Sweden; extraction unit model 1043 and service unit model 1046); ash by incineration in a muffle furnace at 450 °C for 16 h.

The amino acid content of the experimental diets was analyzed using high-performance liquid chromatography (HPLC). For that, samples were hydrolyzed for 23 h with 6N hydrochloric acid at 110 °C under N_2 atmosphere. Samples were pre-column (Waters Pico-Tag column, 4µm, 3.9x300mm) derivatised with phenylisothiocyanate reagent (PITC; Waters WAT088120) before separation by gradient exchange chromatography at 46 °C (Waters auto sample model 717 plus; Waters binary pump model 1525; Waters dual absorbance detector model 2487), according to the Pico-Tag method. Norleucine was used as an internal standard. Chromatographic peaks were identified, integrated and quantified with a Waters Breeze software package by comparing to a known amino acid standard (Pierce NC10180). Cysteine levels were not determined. Trp was measured by a spectrophotometric method as described by De Vries et al. (1980).

For hepatic glycogen content, a portion of liver was homogenized in five volumes of ice-cold distilled water, and glycogen determined by amyloglucosidase hydrolysis following the method described by Roehrig and Allred (1974). Hepatic lipids were determined according to Folch et al. (1957).

2.4.2 Plasma metabolites

Commercial kits from Spinreact, S.A. (Gerona, Spain) were used for determination of glucose (ref: 1001191), total protein (ref: 1001291), triglycerides (TAG; ref: 1001312), and total cholesterol (ref: 1001090).

2.4.3 Enzymatic activity assays

For enzymatic analysis, intestine and liver were homogenized in ice-cold buffer (100mM-Tris-HCl, 0.1mM-EDTA, and 0.1 % Triton X-100 (v/v), pH 7.89) and centrifuged at 30 000g for 30 min at 4 °C. The resultant supernatants were collected and aliquots were stored at -80°C until enzyme analysis. All enzyme activities were measured at 37°C, by monitoring absorbance changes in a microplate reader (ELx808™; BioTek Instruments).

2.4.3.1 Digestive enzyme activities

α-amylase (EC 3.2.1.1) activity was determined with a commercial kit (ref. 41201, Spinreact, Girona, Spain) with modifications; the rate of product formation (2-chloro-4-nitrophenol) was quantified at 405 nm. Lipase (EC 3.1.1.3) activity was determined using

a commercial kit (ref. 1001275, Spinreact, Girona, Spain) with modifications; 1-2-O-dilauryl- rac-glycero-3-glutaric acid-60-methylresorufin-ester was used as substrate, and the formation rate of methylresorufin was followed at 580 nm. Total protease activity was measured by the casein-hydrolysis method. A reaction mixture containing casein at 1% (w/v), buffer (0.1 M Tris HCl at pH 8) and sample was incubated for 1 h at 37°C, stopped by adding trichloroacetic acid solution (8%; w/v); kept for 1 h at 2°C, centrifuged at 1800 g for 10 min and the supernatant absorbance was measured at 280 nm against blanks. A control blank for each sample was assayed by adding the sample supernatant from the homogenates after the incubation time. Tyrosine solution was used as a standard.

2.4.3.2 Intermediary metabolism enzymes

Hexokinase (HK; EC 2.7.1.1) and glucokinase (GK; EC 2.7.1.2) activities were determined as described by Vijayan et al. (1990) and the reaction mixture contained 50 mM imidazole-HCl buffer (pH 7.4), 2.5mMATP, 5mMMgCl2, 0.4mMNADP, 2 units mL^{-1} G6PDH and 1 mM (HK) or 100 mM (HK-IV) glucose. Pyruvate kinase (PK; EC 2.7.1.40) activity was performed with a reaction mixture consisting of 50 mM imidazole-HCl buffer (pH 7.4), 5 mM MgCl2, 100 mM KCl, 0.15 mM NADH, 1 mM ADP, 2 units mL^{-1} LDH and 2 mM PEP (Morales et al., 1990). Fructose 1,6-bisphosphatase (FBPase; EC 3.1.3.11) activity was performed with a reaction mixture consisting of 50 mM imidazole-HCl buffer (pH 7.4), 5 mM MgCl2, 12 mM 2-mercaptoethanol, 0.5 mM NADP, 2 units mL^{-1} G6PDH, 2 units mL^{-1} PGI and 0.5mMfructose 1,6- bisphosphate (Morales et al., 1990). Glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity was measured as described by Morales et al. (1990), using a reaction mixture containing 50 mM imidazole-HCl buffer (pH 7.4), 5 mM MgCl2, 2 mM NADP and 1 mM glucose-6-phosphate. Glutamate dehydrogenase (GDH; EC 1.4.1.2) activity was performed using a reaction mixture containing 50 mM imidazole–HCl buffer (pH 7.4), 0.2 mM NADH, 1 mM ADP, 100 mM ammonium acetate, 2 units mL^{-1} LDH and 10 mM α-ketoglutarate (Morales et al., 1990). Aspartate aminotransferase (ASAT; EC 2.6.1.1) activity was determined as described by Singer et al. (1990) and the reaction mixture contained 50 mM imidazole-HCl buffer (pH 7.4), 10 mM α-ketoglutarate, 0.3 mM NADH, 0.05 mM pyridoxal phosphate, 3 units mL^{-1} MDH and 25 mM L-aspartate. Alanine aminotransferase (ALAT; EC 2.6.1.2) activity was determined as described by Morales et al. (1990) and the reaction mixture contained 50 mM imidazole-HCl buffer (pH 7.4), 10 mM α-ketoglutarate, 0.2 mM NADH, 0.05 mM pyridoxal phosphate, 2 units mL^{-1} LDH and 25 mM L-alanine.

All enzyme activities were expressed as specific activity (mU per mg of soluble protein). One unit (U) of enzyme activity was defined as µmol of product generated per minute under the measurement conditions described above. Protein concentration was determined using Bradford's method (1976), with bovine serum albumin solution as standard.

2.5 Statistical analysis

Data are presented as mean and pooled standard error of the mean (SEM). Statistical analysis of data was done by two-way analysis of variance (Two-way ANOVA) with stocking density and diets as fixed factors. Data were checked for normal distribution and homogeneity of variances and normalized if necessary. The probability level of 0.05 was used for rejection of the null hypothesis, and significant differences among means were determined by Turkey's multiple. All statistical analyses were performed using SPSS 24.0 software package for Mac.

3. Results

Fish promptly accepted the experimental diets at both stocking densities, and mortality during the trial was low and unaffected by diet or stocking density (Table 2). HSD reduced growth performance, voluntary feed intake, feed efficiency, protein efficiency, and N retention (g kg $ABW^{-1}day^{-1}$). However, dietary Trp supplementation did not affect growth performance or feed utilization.

At the end of the trial, whole-body composition was not affected by diet, but whole-body protein was higher and lipids were lower in fish maintained at HSD than at LSD (Table 3). Liver glycogen was higher in fish maintained at LSD and was unaffected by diet composition, while lipids content were higher in fish fed the Trp1 diet than the other diets, and was unaffected by stocking density. Visceral index was not affected by diets or stocking densities, while hepatosomatic index was lower in fish maintained at HSD than at LSD.

Intestinal amylase activity was unaffected by diet or stocking density (Table 4). Lipase and protease activities were also not affected by stock density. Dietary Trp supplementation increased lipase activity, but while in fish maintained at LSD this effect was noticed in fish fed diets Trp1.5 and Trp2, in fish maintained at LSD this effect was only observed in fish fed diet Trp2. Protease activity was higher in fish fed the Trp1.5 diet than the other diets.

Plasma glucose was higher in fish maintained at HSD and decreased as dietary Trp increased (Table 5). On the contrary, total plasma protein, triglycerides, and cholesterol levels were lower in fish maintained at HSD. Total plasma protein was unaffected by diet composition, but plasma triglycerides and cholesterol were higher in fish fed the control than Trp supplemented diets.

Hepatic activity of GK, PK, FBPase, and ASAT was higher in fish maintained at HSD while the opposite occurred for HK and G6PDH (Table 6). Both GK and FBPase activities were higher in fish fed the Trp supplemented diets than the control diet. On the contrary, GDH was lower in fish fed the Trp1.5 diet than the other diets.

Table 2. Growth performance and feed utilization efficiency of gilthead seabream fed the experimental diets.

Density	Low density				High density				*Two-way* ANOVA		
Diet	Trp1	Trp1.5	Trp2	SEM	Trp1	Trp1.5	Trp2	SEM	Density	Diet	Interaction
Initial density (kg m^{-3})	5				16						
Final density (kg m^{-3})	25.4	25.6	26.0	0.70	56.9	55.7	53.9	0.78	***	ns	ns
IBW (g)	11.9	11.9	12.0	0.03	12.0	12.0	12.0	0.00	ns	ns	ns
FBW (g)	57.2	57.6	58.6	0.41	42.7	41.8	40.4	0.65	***	ns	ns
WG (g kg ABW^{-1} day^{-1})[1]	20.5	20.4	20.6	0.05	17.5	17.3	17.0	0.17	***	ns	ns
DGI[2]	2.45	2.45	2.50	0.01	1.88	1.84	1.79	0.03	***	ns	ns
FI (g kg $ABW^{-1}day^{-1}$)[3]	23.6	23.9	24.9	0.38	23.4	20.8	21.6	0.75	**	ns	ns
FE[4]	0.87	0.86	0.83	0.01	0.75	0.83	0.78	0.02	*	ns	ns
PER[5]	1.99	1.97	1.90	0.02	1.72	1.91	1.79	0.05	**	ns	ns
NR (g kg ABW^{-1} day^{-1})[6]	0.44	0.46	0.46	0.01	0.40	0.42	0.40	0.01	**	ns	ns
NR (%NI)[7]	26.7	27.7	26.4	0.86	24.6	28.7	26.4	0.95	ns	ns	ns
Mortality (%)[8]	13.3	11.7	10.0	5.40	10.0	10.6	10.0	3.07	ns	ns	ns

Values presented as means (n = 3) and pooled standard error of the mean (SEM).
Two-way ANOVA: ns: non-significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
ABW: average body weight = (IBW + FBW)/2
Initial body weight
Final body weigh
[1]Weight gain: ((FBW-IBW) × 100) / (ABW* time in days)
[2]Daily growth index: (($FBW^{1/3}$ – $IBW^{1/3}$)/ time in days) × 100
[3]Feed intake
[4]Feed efficiency: wet weight gain / dry feed intake
[5]Protein efficiency ratio: wet weight gain / crude protein intake
[6]Nitrogen retention (g kg $ABW^{-1}day^{-1}$) = (FBW×FBN−IBW×IBN)/((IBW+FBW)/2IBW×time in days)
[7]Nitogen retention: (% nitrogen intake) = (FBW×FBN−IBW×IBN)/(NI))×100
[8]Mortality

Table 3. Whole-body composition (% wet weight), hepatosomatic and visceral index and liver composition of gilthead seabream juveniles fed the experimental diets.

Density	Initial	Low Density				High Density				*Two-way* ANOVA					
Diet		Trp1	Trp1.5	Trp2	SEM	Trp1	Trp1.5	Trp2	SEM	Density	Diet	Interaction	Trp1	Trp1.5	Trp2
Dry Matter (%)	24.0	22.1	22.1	22.7	0.31	22.9	23.6	22.9	0.21	ns	ns	ns			
Protein	13.9	13.5	14.0	13.9	0.20	14.1	14.8	14.5	0.18	*	ns	ns			
Lipids	5.2	8.3	8.7	8.7	0.92	7.9	7.5	7.8	1.26	*	ns	ns			
Ash	6.1	3.3	3.9	3.4	0.75	3.8	3.9	3.7	0.18	ns	ns	ns			
Liver Lipids	-	6.17	5.16	5.92	0.48	6.69	5.87	5.20	0.46	ns	***	ns	b	a	a
Liver Glycogen	-	58.4	61.2	60.1	1.47	47.8	49.5	52.8	1.60	***	ns	ns			
HSI[1]	-	1.3	1.4	1.4	0.03	1.1	1.1	1.2	0.03	***	ns	ns			
VI[2]	-	13.1	12.3	12.7	0.25	13.0	12.2	13.3	0.34	ns	ns	ns			

Values presented as means (n = 3; n=9 for liver analysis) and pooled standard error of the mean (SEM).
Two-way ANOVA: ns: non-significant (p > 0.05); *p < 0.05; **p < 0.01; ***p < 0.001.
[1]Hepatosomatic index: (liver weight/body weight)×100
[2]Visceral index: (viscera weight/body weight)×100

Table 4. Specific activity (mU mg protein^{-1}) of intestinal amylase, lipase, and proteases of gilthead seabream juveniles fed experimental diets.

Density	Low Density				High Density				*Two-way* ANOVA					
Diet	Trp1	Trp1.5	Trp2	SEM	Trp1	Trp1.5	Trp2	SEM	Density	Diet	Interaction	Trp1	Trp1.5	Trp2
Amylase	23.0	26.5	24.7	1.61	22.1	25.4	21.7	1.41	ns	ns	ns			
Lipase	0.19^{aA}	0.25^{b}	0.20^{bA}	0.12	0.22^{aB}	0.23^{a}	0.31^{bB}	0.02	ns	*	*			
Protease	34.7	40.1	34.3	0.81	36.9	38.7	36.0	0.50	ns	***	ns	a	b	a

Values presented as means (n = 9) and pooled standard error of the mean (SEM).
Two-way ANOVA: ns: non-significant (p > 0.05); *p < 0.05; **p < 0.01; ***p < 0.001. If interaction was significant, density and dietary Trp effects were analysed by t-Test or one-way ANOVA, respectively; means in the same line with different capital and small letters indicate significant differences (P < 0.05) for density or Trp levels, respectively; means with no letters are not significantly different (P > 0.05).

Table 5. Plasma metabolites concentration (mg dL^{-1}) of seabream fed the experimental diets.

Density	Low Density				High Density				*Two-way* ANOVA					
Diet	Trp1	Trp1.5	Trp2	SEM	Trp1	Trp1.5	Trp2	SEM	Density	Diet	Interaction	Trp1	Trp1.5	Trp2
Glucose	112.1	93.9	93.7	3.63	147.8	129.7	115.6	5.3	***	**	ns	b	ab	a
Total Protein	3.58	3.40	3.48	0.06	3.27	3.09	3.10	0.06	***	ns	ns			
Triglycerides	328.7	269.1	265.5	11.5	239.8	197.3	208.4	8.66	***	*	ns	b	a	a
Cholesterol	268.9	232.0	224.6	6.74	215.9	188.3	202.8	4.20	***	***	ns	b	a	a

Values presented as means (n=9) and pooled standard error of the mean (SEM).
Two-way ANOVA: ns: non-significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 6. Specific activity of hexokinase (HK), glucokinase (GK), pyruvate kinase (PK), fructose-1.6-bisphosphatase (FBPase), glucose-6-phosphate dehydrogenase (G6PDH), glutamate dehydrogenase (GDH), alanine aminotransferase (ASAT) and aspartate aminotransferase (ALAT) (mU mg $protein^{-1}$) in liver of seabream fed the experimental diets.

Density	Low Density				High Density				*Two-way* ANOVA					
Diet	Trp1	Trp1.5	Trp2	SEM	Trp1	Trp1.5	Trp2	SEM	Density	Diet	Interaction	Trp1	Trp1.5	Trp2
Glycolysis														
HK	3.8	3.5	3.9	0.12	3.3	3.1	3.3	0.10	***	ns	ns			
GK	3.9	5.0	4.9	0.13	4.5	5.4	5.4	0.12	*	***	ns	a	b	b
PK	10.7	10.6	10.5	0.58	13.5	13.6	13.2	0.80	**	ns	ns			
Gluconeogenesis														
FBPase	45.1	53.3	53.5	1.68	52.4	57.2	56.5	1.63	**	***	ns	a	b	b
Lipogenesis														
G6PDH	139.6	129.1	135.3	8.63	112.9	113.5	98.5	9.01	*	ns	ns			
Amino acid catabolism														
GDH	362.9	290.8	345.5	13.0	342.5	294.7	365.3	8.25	ns	**	ns	b	a	b
ALAT	381.3	329.3	437.7	31.7	359.1	345.1	291.2	8.23	ns	ns	ns			
ASAT	426.8	360.2	436.0	24.10	454.6	452.0	525.3	9.54	*	ns	ns			

Values presented as means (n=9) and pooled standard error of the mean (SEM).
Two-way ANOVA: ns: non-significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

4. Discussion

Keeping fish at high stocking density is a common practice in intensive aquaculture to increase production per unit of water volume. However, depending on intensity and exposure time, rearing fish at high density may cause specific injuries or switch metabolic and energetic pathways to compensate for the stress-induced effect (Tort et al., 2011). The persistence of response to chronic stress may lead to the exhaustion of nutrient resources, followed by malfunction of key regulatory systems, such as osmoregulation or immune competence (Tort et al., 2011). Growth depression is common in fish kept at HSD and it has been attributed to the elicited stress response, involving primary (hormonal), secondary (metabolic alteration), and tertiary (voluntary feed intake, growth) responses, as well as to social interactions (Montero et al., 1999; Ibarz et al., 2005; Conde-Sieira et al., 2010; Tejpal et al., 2014; Di Maggio et al., 2014).

For gilthead seabream, evidence has shown that HSD may be considered a chronic stressor that induces stress responses and impairs fish growth, well-being, and health (Montero et al., 1999; Rotllant et al., 2001; Sangiao-Alvarellos et al., 2005, Mancera et al., 2008). Increased plasma cortisol (primary response) has previously been reported for gilthead seabream kept at HSD (Montero et al. 1999; Sangiao-Alvarellos et al., 2005; Alves et al., 2010). Results of the present study further indicate that the HSD induced chronic stress in gilthead seabream, as reflected by the reduction of growth and feed intake (tertiary responses).

The effect of HSD on gilthead seabream performance is also related to fish weight. For instance, Canario et al. (1998) compared performance of 1.3 g fish kept at initial stocking densities of 0.35, 1.3, and 3.2 kg/m³ (and reaching final stocking densities of 2.4, 8.1 and 16.1 kg/m³), and concluded that fish at the highest stocking density grew 25% lower than fish at the lowest density. However, Montero et al. (1999) observed that initial stocking densities of 2.6 and 10 kg/m³ (and reaching final stocking densities of 10 and 40.8 kg/m³ and final weight of 82-85g) did not affect growth and feed intake of fish with an initial weight of 22 g. In the present study, fish weight was within the range of that of Montero et al. (1999) study. Thus, it can be assumed that fish kept at LSD (5-26 kg/m³) were not restrained in terms of growth performance. However, growth of fish kept at HSD (16-57 kg/m³) was negatively affected. Based on ours and Montero et al. results, it can be suggested that 40 kg/m³ is near the maximum tolerable stocking density for gilthead seabream of the weight range tested.

This is further reinforced by the plasma glucose level, which is an indicator of secondary stress response in fish (Tort et al., 2011). In the present study, plasma

glucose level of fish kept at HSD was higher than that of fish kept at LSD, indicating that fish at HSD were under a chronic stress condition. Similar results were previously reported by other authors for fish kept at HSD conditions (Sangiao-Alvarellos et al., 2005; Di Marco et al., 2008; Alves et al., 2010; Herrera et al., 2012; Costas et al., 2013).

Rearing density did not affect digestible enzymes activity of gilthead seabream, suggesting that diet digestibility was also not affected. However, rearing density affected intermediary metabolism and energy subtracts utilization. Fish maintained under crowding stress conditions had lower plasma protein, triglycerides, and cholesterol levels, increased liver glycolysis and gluconeogenesis and decreased lipogenesis. Overall, these biochemical and metabolic alterations were also reflected in lower N retention (g kg ABW^{-1} day^{-1}), whole-body lipid, and liver glycogen contents of fish reared at HSD, indicating an increased mobilization of energy in gilthead seabream kept in crowding conditions. This is in line with the increased energy demand previously reported for gilthead seabream (Montero et al., 1999; Ibarz et al., 2005, 2007) and other species (Morales et al., 2005; Das et al., 2009) subjected to different stressors.

The adaptive response of gilthead seabream to the stress induced by HSD involved an increase of glycogenolysis and gluconeogenesis, and led to a decrease of liver glycogen content. This increased glucose export capacity of liver was probably required to maintain the higher plasma glucose levels in HSD group. A similar pattern was also observed for gilthead seabream maintained at very high stocking density (70 kg/m^3) for 14 days (Sangiao-Alvarellos et al., 2005). Also in gilthead seabream kept under chronic stress, Alves et al. (2010) observed an upregulation of gene encoding for the enzymes involved in liver glycogenolysis and gluconeogenesis and of lipid mobilization. In the present study, mobilization of liver lipids did not seem to have occurred, as liver lipid content was not affected by density. Instead, carbohydrates seemed to be mobilized as major energy substrate, as suggested by the decrease of liver glycogen content.

G6PDH catalyzes the first committed step of the pentose phosphate pathway, which is involved in the generation NADPH that is pivotal in lipid synthesis. In the present study, G6PDH activity was decreased in fish maintained at HSD suggesting a decrease of lipogenesis, which is in agreement with the decreased whole-body lipid content of fish maintained at HSD. Similarly, a down-regulation of the gene encoding for G6PDG was observed in gilthead seabream (Alves et al., 2010) and rainbow trout (Vijayan et al., 1990) maintained at HSD.

Even though evidences of increased protein catabolism in fish under stressed conditions are limited, some authors have observed an increase of amino acid catabolic enzymes activity (Van der Boon et al., 1991. Morales et al., 2005). In the present study, ASAT (but not GDH and ALAT) activity was increased in fish maintained at HSD. This indicates a possible increase of protein used for energetic purposes and is in line with the decreased protein retention (g kg ABW^{-1} day^{-1}) observed in the HSD group.

To the best of our knowledge, no data is available on the potential of DDGS as ingredient in gilthead seabream diets. In the present study, fish performed well and growth rate and feed utilization were similar or higher than that observed in other studies with gilthead seabream fed FM-based diets (Couto et al., 2008; Peres et al., 2011, 2013; Coutinho et al., 2016a, b; Kokou et al., 2016; Monge-Ortiz et al., 2016). This, despite the low fish meal (15%) and high DDGS (30%) levels of the experimental diets.

One of the possible limitations of DDGS use in aquafeeds is its low Trp level and the low Trp:BCAA ratio, which may reduce transport of Trp through the blood-brain barrier, reducing the synthesis and the release of serotonin (Fernstrom, 2005). Indeed, Trp is the only precursor of serotonin and melatonin (Lepage et al., 2005; Martins et al., 2013), which are known to be implicated in the control of agonistic behavior, stress responses, endocrine functions, antioxidant and immune responses in animals (Winberg and Thörnqvist, 2016; Hoseini et al., 2017). Thus, limited Trp availability may be a concern, particularly under chronic stress conditions. Indeed, dietary Trp supplementation was shown to have stress-reducing effects in different fish species (Lepage 2002; 2003, Hoseini et al., 2012; Basic et al., 2013, Kumar et al., 2014; Hoseini et al., 2017). However, in this study dietary Trp supplementation had no effect on growth performance, indicating that Trp level of the control diet was not limiting. This further confirms that the dietary Trp recommendation indicated by Peres and Oliva-Teles (2009) adequately meets the requirement of this amino acid for gilthead seabream juveniles.

High levels of Trp may increase synthesis and release of serotonin and melatonin (Lepage et al., 2005; Martins et al., 2013) and this may reduce voluntary feed intake (Rubio et al., 2004; Lopez-Olmeda et al., 2006). Further, excess of Trp may impair growth and feed intake in terrestrial animals (Edmonds and Baker, 1987; Chung et al., 1991) and fish (Santiago and Lovell, 1988; Borlongan, 1991; Ahmed, 2012; Tang et al., 2013; Farhat and Khan, 2014; Wen et al., 2014). In this study, although dietary Trp was supplemented up to 2 times the estimated requirement for gilthead seabream, this did not affect growth performance or feed intake, suggesting that up to this dietary level, Trp is within the safety margin for dietary inclusion of this amino acid.

Little is known regarding the effect of serotonin, melatonin and its precursor, Trp on fish digestive processes. In rat, in vivo and in vitro studies showed that the stimulatory effects of melatonin or Trp on the exocrine pancreas activity involves cholecystokinin release (Jaworek et al., 2004), which is known to be a regulator of pancreatic enzyme secretion in fish (Aldman et al., 1992; Murashita et al., 2008). In this study, dietary Trp supplementation seems to have modulated digestive enzymes activity, namely by increasing proteases and lipase activities. This effect may be attributed to higher release of melatonin, a hormone involved in the regulation of the digestive process (Falcon et al., 2010; Muñoz-Pérez et al., 2016). Indeed, oral or dietary melatonin supplementation was shown to upregulate digestive enzymes activity in fish (Conde-Sieira et al., 2014; Pianesso et al., 2015). However, an inhibitory effect of digestive enzymes activity in fish fed diets supplemented with Trp or melatonin was also observed (Mardones et al, 2018).

Plasma triglycerides and total cholesterol were reduced with in fish fed Trp supplemented diets. As feed intake was not affected, and lipase activity was increased by dietary Trp supplementation, these results cannot be attributed to reduced cholesterol and triglycerides load after digestion. A depressive effect of melatonin on plasma cholesterol and triglycerides levels was previously reported in Nile tilapia (Singh et al., 2012), and this may explain the observed effect.

As aforementioned, plasma glucose level is one of the most common secondary stress response parameter tested and its increase is often associated to an elevation of plasma cortisol of fish under stress conditions (Tort et al., 2011). In present study, dietary supplementation with Trp decreased plasma glucose, highlighting a stress mitigation effect of Trp. Further, plasma glucose level reduction was more important in HSD groups, denoting an accentuated effect of Trp in relieving stress in these fish. Indeed, in fish kept at HSD and fed the Trp2 diet plasma glucose levels were similar to those of LDS fish fed the control diet. Similarly, dietary Trp supplementation reduced plasma glucose levels of *Cirrhinus mrigala* kept in HSD or LSD (Tejpal et al., 2009), as well as in other species submitted to different stressors (Hoseini et al., 2012; Kumar et al., 2014). Also, oral melatonin supplementation had no effect on plasma glucose levels in non-stressed rainbow trout, whereas in chronic stressed fish melatonin decreased plasma glucose to levels below those obtained for non-stressed fish (Conde-Sieira et al., 2014).

Hepatic GK and FBPase increased with dietary Trp up to 1.5 times the basal level. The underlying mechanisms of Trp on glucose metabolism are not well understood, but changes in melatonin and serotonin synthesis may be involved

(Keszthelyi et al., 2009). In terrestrial animal, it was reported that dietary Trp supplementation can increase insulin sensitivity, probably through increased serotonin synthesis (Ponter et al., 1994), and this may result in reduction of plasma glucose levels (Yamada et al., 1989; Sugimoto et al., 1990). A similar effect in gilthead seabream would contribute to explain the increased GK activity in fish fed Trp supplemented diets. The apparent contradictory responses of GK and FBPase activities cannot be explained at this point. However, it is known that fish do not regulate well glucose metabolism, and in gilthead seabream it was observed that an intraperitoneal glucose load increased glycolic enzymes activity but did not reduce the activity of gluconeogenic enzymes (Enes et al., 2009).

From this study, it can be concluded that high stocking density (final density >55 kg m^{-3}) decreased growth performance and feed intake of gilthead seabream without affecting feed efficiency. At HSD, gilthead seabream readjusted energy metabolism and decreased energy reserves, including whole-body lipid and liver glycogen levels. Irrespective of rearing density, dietary Trp supplementation did not affect growth and feed efficiency but seemed to mitigate stress of fish kept at HSD. Further studies are however necessary to further elucidated the role of dietary Trp surplus on stress response of gilthead seabream.

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Chapter 6

Final considerations

6.1 General conclusions

Under the actual panorama of increased demand and limited availability of FM, researchers have been centering efforts to search for low-cost alternative protein sources with renewed value. Oven the last two decades, plant protein feedstuffs have been used to replace FM to a great extent in diets for carnivore fish species. SBM has been the most widely used alternative protein source and it has been successfully incorporated in carnivorous diets at variable levels. However, besides the well-known nutritional limitations of using SBM in carnivorous fish diets, SBM use in aquafeeds poses social and environmental sustainability issues, besides economical ones. For example, "food-feed competition", rising prices, and carbon footprint involved in importing SBM from major exporting markets (USA and Brazil) to Europe, have to be considered. Under this context, the use of locally available agro-industrial plant and animal by-products as aquafeeds ingredients seems to be promising. DDGS is a by-product of ethanol production, moderately high in protein and competitively priced relative to other alternative protein sources. However, critical science and knowledge gaps hinder the DDGS incorporation in carnivorous fish diets. Thus, it is of upmost importance to study the nutritional potential of DDGS and to develop strategies to overcome some of the nutritional limitations of its use in carnivorous fish feeds.

DDGS used in the preset study was produced in Europe by Pannonia Gold, being a non-GM DDGS. The DDGS protein and lysine content level (protein content: 32.8%; lysine content: 1.24%DM) were within the higher range of reported DDGS composition (protein content: 26.3 to 34.0%; lysine content: 0.29 to 1.24%). Besides its moderate lipids level (fat content: 9.0%), DDGS used in this study was also high in fiber (neutral detergent fiber: 42.3; acid detergent fiber: 14.4%; starch: 0.5%). Values were however within reported values for other DDGS sources (fat: 8.8 to 18.5%; neutral detergent fiber: 26.5 to 54.1%; starch: up to 5.1%; Spiehs et al., 2002; Batal and Dale, 2006; Øverland et al., 2013Belyea et al., 2004).

As expected, due to the strictly carnivorous nature of turbot, the potential of DDGS as alternative ingredient seems to be higher for gilthead sea bream than that of turbot. However, a direct comparison between both species cannot be done as different approaches were used in this thesis. For turbot, DDGS was used to replace dietary FM while for gilthead seabream DDGS was used to replace dietary SBM. For turbot juveniles, results of the present study indicate that DDGS was not well utilized and that even a dietary inclusion of 10%, replacing just 8% of FM protein, negatively affected

growth performance and overall feed efficiency. In this study, FM level of experimental diets was reduced from 40% to 32.2% with the inclusion of increased levels of DDGS, and this may also have contributed to the results. Levels of FM replacement by alternative feedstuffs in turbot diets seem to be dependent on FM level in the control diet. Indeed, Bonaldo et al. (2011) concluded that by replacing 10% of FM by a mixture of plant feedstuffs in a diet with 55% FM just slightly, but not significantly, reduced growth performance, while replacing 15% of FM by a mixture of plant feedstuffs in a diet with 50% FM significantly reduced growth and feed utilization (Bonaldo et al., 2015). Dong et al. (2016) also observed that dietary FM reduction from 62 to 40% by replacement with a mixture of plant and animal ingredients did not reduce turbot growth performance, while dietary FM reduction to 31% significantly affected fish performance. On other hand, for gilthead seabream juveniles, the inclusion of up to 35% DDGS replacing SBM at a constant FM level (35% DM) did not affect growth performance, feed efficiency, protein and energy retentions and tended to decrease the cost of fish production (€ per kg of fish produced). The dietary DDGS level successfully achieved in this study for gilthead sea bream is higher than that considered acceptable for other carnivorous fish. Indeed, for carnivorous fish, maximum dietary DDGS inclusion was estimated to be 10-20% for rainbow trout (Welker et al., 2014b), 14-21% for olive flounder (Rahman et al., 2015), but high DDGS incorporation levels are tolerated by omnivorous species. This opens a great potential of reducing the imports of SBM in diets for such an important aquaculture species in Europe, thus reducing feed costs and carbon footprint, and increasing the use of local available feedstuffs.

Palatability may be also an issue when considering dietary FM replacement in aquafeeds, as alternative feedstuffs may not be so palatable and therefore voluntary feed intake may be compromised, which in turn may affect growth performance. In this study, for both species voluntary feed intake, expressed per unit body weight basis (g kg $ABW^{-1}day^{-1}$), was not affected by the dietary inclusion of DDGS. Also for both species, the whole-body composition was not affected by dietary DDGS inclusion, except for lipids and energy content, which seemed to be related to the lower performance of DDGS-based diets.

For turbot, dietary inclusion of DDGS did not affect feed intake, and therefore differences in growth performance and overall feed efficiency seem to have resulted of poorer diet digestibility or metabolic utilization. Indeed, apparent digestibility coefficients (ADCs) of energy of DDGS-based diets decreased with the increase of dietary DDGS level, while the ADC of protein and amino acids were similar among diets, as previously

reported by other studies (Øverland et al., 2013; Welker et al., 2014b; Magalhães et al., 2015). In accordance, with the increase of dietary DDGS inclusion, activities of the digestive enzymes amylase and lipase, but not protease, were reduced. These results may be related to the increased NSP levels in DDGS diets, which in high trophic level species are known to impair dry matter and energy digestibility (Tibbetts et al., 2006; Nagel et al., 2012; Magalhães et al., 2015) and reduce intestinal trypsin and amylase activities in turbot (Hu et al., 2015). In the present study, lipase and proteases activities, but not amylase, increased along the intestine, irrespective of treatments. This is probably due to chyme drag along the intestine or to higher role of posterior intestine in the digestion process, as suggested by Izquierdo and Henderson (1998).

Plasma metabolites profile reflect somehow the feed utilization efficiency. In turbot, dietary DDGS level reduced plasma total protein, albumin, triglycerides, and cholesterol. In line with the growth performance results, this may be indicative of deficient nutrient uptake, as levels of plasma proteins, including total protein, albumin, and transferrin have been proposed as markers of protein malnutrition in turbot (Nagel et al., 2012) as well as in other fish species (Peres et al., 2013, 2014). This seemingly protein malnutrition may have also impaired the export of triglycerides and of other lipids from the liver, decreasing plasma triglycerides and cholesterol levels. Contrarily, in gilthead seabream, and in line with the growth performance data, dietary SBM replacement by DDGS did not affect plasma glucose, protein, albumin, and globulins levels but increased plasma triglycerides and decreased cholesterol levels. It has been reported that yeast or yeast-derived products may affect plasma lipid profile, decreasing plasma cholesterol (Kumar et al., 2013; Øverland et al., 2013) or, in opposition, increasing cholesterol and decreasing triglycerides (Mohebbi et al., 2013), and so these results may be due to the presence of yeast cells component in DDGS, which is approximately 4% (Ingledew, 1999).

For both species, the specific activity of amino acid catabolic key enzymes correlated relatively well with the overall protein utilization efficiency data, increasing in turbot but decreasing in gilthead sea bream. In both species, the activity of key enzymes of glycolysis, gluconeogenesis, and lipogenesis was not affected by the dietary inclusion of DDGS, with the exception of fructose-1,6-bisphosphatase for gilthead seabream, a gluconeogenic enzyme which activity decreased with the increase of DDGS level in the diet.

In turbot, dietary inclusion of DDGS did not affect oxidative stress susceptibility in both intestine and liver. Even though, higher oxidative damage was observed in the intestine than in liver, which may be associated with higher antioxidant capacity in the liver than in the intestine or with higher diet-induced oxidative damage in the intestine. Indeed, the intestine is the first organ to be challenged by a number of nutritional insults; it is mostly likely that a negative response to dietary nutrients will appear in this organ.

Contrarily, in gilthead sea bream an overall reduction in the activity of counter-acting oxidative stress enzymes was observed, leading to increased liver lipid peroxidation with the DDGS-based diets. The reason for the increased susceptibility to oxidative stress in fish fed DDGS-based diets is not clear, requiring further studies, particularly regarding the presence of secondary lipid oxidation products originated during DDGS technological process (Han and Csallany, 2008).

As aforementioned, one of the constraints of using plant feedstuffs as alternative to FM is the presence of antinutritional factors. DDGS lacks anti-nutritional factors, except for the relatively high level of NSP. Besides representing an indigestible energy fraction, as fish lack of specific digestive non-starch polysaccharidases, NSP may impair overall diet digestibility, oxidative status, and immune functions of the intestine (Sinha et al., 2011). Thus, dietary supplementation of DDGS based-diets with exogenous enzymes was tested as a strategy to improve its nutritional value. For turbot, it was observed that exogenous enzyme complexes increased the ADC of dry matter, protein, lipid, energy and of some EAA. Further, the activity of digestive enzymes was also increased as well microbiota richness and diversity. For gilthead seabream exogenous enzyme complex increased overall feed efficiency, nitrogen and energy retentions, and counteracted the increased susceptibility to hepatic oxidative stress induced by DDGS. Moreover, it reduced feeding costs per kg of fish produced by 16.6%. Together, these results suggest that the tested exogenous enzymes complex reduced digesta viscosity and increased enzyme access to subtracts. Nevertheless, the different results obtained with the two exogenous enzyme complexes showed the pivotal importance of understanding the potential and limitations of exoenzymes, as if inappropriately applied it will lead to waste of resources.

Differences between species and exogenous enzymes supplements highlight the need to carefully evaluate the benefits of different exoenzymes complexes according to species, diet formulation and enzyme supplementation level. Moreover, besides species-specific differences in digestive machinery capability, differences in rearing temperature

may also contribute to explain the differences in exoenzymes efficacy between turbot and gilthead sea bream. Indeed, the optimum temperature of activity of exogenous enzymes is closer to that of rearing water temperature of gilthead seabream (22-25 ºC) than that of turbot (18ºC).

Another constrain to the use of DDGS as alternative ingredient, is the low tryptophan (Trp) to high branched-chain amino acids (BCAA) ratio that may reduce the intestinal absorption of Trp and thus induce Trp deficiency. Therefore, it was hypothesized that high incorporation level of DDGS might impair the capacity of gilthead seabream juveniles to cope with chronic stress induced by high stocking density, as Trp is the precursor of the synthesis of the neurotransmitter serotonin. Irrespective the experimental diet, stocking density reduced growth performance and feed intake, but did not compromise feed efficiency, suggesting that high density induced a chronic stress. However, irrespective of stocking density, supplementation of diets with Trp did not affect growth and feed efficiency, indicating that dietary Trp level of the control diet was not limiting for serotonin synthesis. Nonetheless, dietary Trp supplementation restored plasma glucose levels of fish kept at high stocking density to levels similar to that of fish kept at low stocking density, suggesting that it had a role in stress mitigation. However, dietary Trp supplementation increased hepatic lipase activity and reduced liver lipids, plasma triglycerides and cholesterol levels, and hepatic activity of key-enzymes of amino acid catabolism, which may be attributed to higher release of melatonin, a hormone involved in the regulation of digestive process (Falcon et al., 2010; Muñoz-Pérez et al., 2016).

Overall, this thesis demonstrated the high potential of DDGS as alternative to traditional plant feedstuffs, as SBM, for gilthead seabream juveniles. For this species, total replacement of soybean meal by DDGS did not compromise growth performance and feed utilization, while slightly decreased feeding cost per kg of fish produced. Concomitant supplementation of DDGS diets with exogenous carbohydrases complex improved feed efficiency and reduced the feeding cost per kg of fish produced. Moreover, exogenous carbohydrases (NAT) supplementation counteracted the increased susceptibility to hepatic oxidative stress induced by DDGS. On the contrary, for turbot the potential of DDGS to replace FM seems to be limited, as dietary inclusion of just 10% DDGS to a 40% FM based diet reduced growth performance. Similarly, to gilthead sea bream, diet supplementation with exogenous enzymes seems to be promising for turbot, increasing digestibility of dry matter, protein, energy and some EAA, the activity of digestive enzyme activities and the richness and diversity of microbiota.

6.2 Future Prospects

Successful completion of this study will allow the aquafeed industry to equate the possibility of incorporating DDGS in marine fish diets, namely for gilthead sea bream, thus reducing dependence on more expensive ingredients, improve feed formulations and decrease feed costs for marine fish species. For new ingredients evaluation, one of the first steps is to determine its nutritional quality through feeding studies, evaluating important aspects as productive performance, digestive capacity and whole-body or fillet composition. However, additional criteria including the modulation capacity of the well-being and health status of fish must also be considered. Due to the presence of yeast as well as the NSP fraction, DDGS may have probiotic or prebiotic effects that deserve to be further explored. In fact, yeast cells have many biologically active compounds with potential immunological effect, including beta-glucans, mannan-oligosaccharides, chitin, nucleotides, and glutamate. Also, oligosaccharides present in the NSP fraction may act as prebiotic, thus modulating gut microbiota community and also having immunological properties. Therefore, dietary incorporation of DDGS may contribute to boosting the fish immune status and diseases resistance, giving an extra value to this ingredient.

Thus, particularly for gilthead seabream, further studies are required to determine if dietary inclusion of DDGS may improve the immunologic state and fish susceptibility to infections and chronic or acute stress. Moreover, due to the high content of NSP in DDGS, the integrity and immune functions of the intestinal epithelium may be impaired. To understand the effects of dietary DDGS inclusion additional studies are needed focusing on health and function status of the digestive tract. Histological analysis may thus be considered in parallel to the study of the intestine immune status, evaluated through to the study of transcriptomic and cell-mediated immune responses.

Another aspect that cannot be disregarded is the potential of DDGS to eventually affect quality traits of the final product. Depending on the technological processing, DDGS may have relatively high level of pigments (lutein and zeaxanthin) that may be deposited as visible yellow pigments in the fillets and that may reduce fish commercial value, and this needs to be evaluated.

Due to the potential of dietary exogenous enzymes supplementation determined in this work, further studies are required to evaluate further their potential in other feed formulations.

For turbot, it would be important to evaluate the potential of DDGS to replace traditional plant feedstuffs, such as SBM, rather than FM, in line with the work conducted with gilthead sea seabream. For gilthead sea bream, since dietary DDGS was well tolerated it would be of interest to study the potential of its use in diets with even lower FM level.

Chapter 7

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